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HETEROKARYOSIS IN ASPERGILLUS

By Kenneth B. Raper and Dorothy I. Fennell

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Introduction

Gossop, Yuill, and Yuill (1940) reported the production of heterokaryons when different mutants, or parent and mutant strains, of *Aspergillus niger* van Tieghem and *A. nidulans* (Eidam) Winter were cultivated in intimate association. For example, from a mixed culture of brown and cinnamon-colored mutants of *A. niger*, they observed the development of brown and cinnamon-colored conidial heads characteristic of the parent types, and, in addition, a third type of spore head, almost black, which developed near the junction of sectors representative of the two paired mutants. Upon recultivation by needle transfer (multiple spores), the cinnamon heads yielded all cinnamon-colored colonies, and the brown heads brown colonies, whereas the third type yielded some cinnamon-colored, some brown, and some mixed colonies in which again cinnamon, brown and black heads could be identified. The latter type was interpreted as representing a heterokaryotic fructification which had developed as the result of hyphal anastomoses with subsequent nuclear intermixture of the two mutant types. No explanation was offered for the dark color of the heterokaryotic heads.

Heterokaryotic heads produced in colonies containing a black parent and a mutant strain of *A. niger* revealed spore chains of two types, one deeply pigmented, and the other almost colorless. When strains of limited color contrast were intermixed, heterokaryotic heads appeared more nearly homogeneous. Mixed white and green heads were observed to result from the joint inoculation of a typical green-spored *A. nidulans* and a white mutant, *A. nidulans* mut. *alba* Yuill (1939), of the same strain. Heterokaryotic heads in this mixture were clearly composed of entire chains of white conidia and similar chains of green conidia, the two types appearing in any proportion in different heads.

In all cases, single spores from heterokaryotic heads gave rise only to pure colonies identical with one or the other of the intermixed strains. Basing their conclusions upon published literature and the results obtained in their experiments, they correctly assumed the conidia to be uninucleate.

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More recently, in a paper which deals primarily with heterokaryons in *Penicillium notatum* Westling, Pontecorvo (1946) reported the production of heterokaryons from paired mutants of *A. oryzae* (Ahlburg) Cohn, and presented further work by the Yuill brothers, including one of their exquisite photographs which illustrates a heterokaryotic head of *A. tamarii* Kita with tan and light-colored spore chains.

Results which confirm and extend appreciably the work of these English workers with *Aspergillus* have been obtained in our Laboratory, primarily from strain intermixtures of *A. fonsecaeus* Thom and Raper (1945), and a preliminary report (abstract) was published in 1948 by Raper and Fennell. The writers regret that the recurring urgency of other work has precluded the earlier publication of this full report of investigations which were initiated more than a decade ago.

METHODS AND CULTURES

Cultivation

Culture media employed most commonly in the investigation included: Czapek's solution agar with 3% sucrose as carbon source and 0.3% sodium nitrate as nitrogen source (Czapek agar); Czapek's agar supplemented with 1% concentrated corn steep liquor (steep agar); Czapek's agar with 20% instead of 3% sucrose (high-sugar agar); and various other modifications of the Czapek formula to obviate nutritional deficiencies of certain mutants and strains. (For details of media composition and preparation, see Raper and Thom, 1949, pp. 64–70.)

Following irradiation with ultraviolet light, conidia were plated at appropriate dilutions in Czapek and steep agars, using 150 mm. diam. Petri dishes to secure maximal colony development. Incubation was at room temperature and plates were carefully screened for mutant heads with a low-power binocular microscope after a period of 10 days to 2 weeks. Mutants were isolated by removal of conidia from such heads, followed by direct transfer to fresh media if the heads were well separated, or by dilution plating if the mutant heads stood in juxtaposition to others. The capacity of two mutant strains, or a mutant and its parent, to form heterokaryons was generally tested by planting conidia of single strains at diagonally opposite corners of an imaginary square (*ca.* 1 cm.) situated at the center of an agar plate. Steep agar was usually employed as the substrate. In plates of this type the opposed strains usually formed plane, well-defined colonies, or sectors, yielding four colony interfaces for heterokaryon development (Fig. 2A). In a few cases, heterokaryons developed only when the opposed strains were planted together as a point inoculation in plates of Czapek or high-sugar agars, this type of seeding apparently effecting a more intimate intermixture of the two mycelia. Heads believed to represent heterokaryons were analyzed by simple streaking of conidial suspensions in cases where only confirmatory evidence was desired. In the majority of cases, however, dilution platings of such suspension were made, using 150 mm. diam. Petri dishes for reasons already cited. In some critical experiments, single conidia were isolated, using the method described by Thom and Raper (1945).

Stock cultures, mutants derived from them, and strain intermixtures containing abundant heterokaryotic heads have been successfully preserved by lyophilization, using the methods reported by Raper and Alexander (1945).

Irradiation

With the single exception of the tan mutant of NRRL 67 (see below), all mutants employed in this investigation were produced by exposing conidia to radiation from germicidal lamps. The apparatus generally used was that employed by Raper and Fennell (1946) for producing mutations in *P. chrysogenum*, and consists of a 4-Watt germicidal lamp of bent tube construction and six inches in length which emits 95% of its effective radiation at wavelength 2537 Å (500 milliwatts). The light source was supported 4 inches above an aqueous suspension of conidia (15 ml.) which was contained in a flat-bottomed Petri dish (100 mm. diam.). The suspension was continually stirred throughout the period of radiation. Preparatory to irradiation, conidia were suspended in sterile water containing sodium lauryl sulfonate (1:10,000 parts) as a wetting agent, filtered through loose cotton tampons to remove spore chains and clumps, and diluted as necessary to yield concentrations in the neighborhood of 5–10 million spores/ml. as determined by haemocytometer counts. The final suspensions were slightly opaque. One-ml. samples of the suspensions subjected to irradiation were removed periodically from 0 (control) to 45 minutes in the case of black-walled conidia and from 0 (control) to 30 minutes, for orange-colored conidia. These samples were plated at appropriate dilutions in Czapek and in steep agars. Following incubation, mutants were isolated in the manner subsequently described.

Staining

A number of cytological techniques were used to elucidate the nuclear picture of the mycelia, conidiophores, vesicles, sterigmata and conidia of *A. fonsecaeus* and other species examined in the study. These included paraffin sections stained with Heidenhain's iron-alum haematoxylin, aceto-carmine smears, and a haematoxylin in propionic acid stain recommended and demonstrated to us by Professor W. N. Stewart, University of Illinois. The last-named stain, which is used in the same general way as aceto-carmine, proved most useful.

Origins and Characteristics of Strains

Stock: A. fonsecaeus Raper and Thom, type strain NRRL 67. Culture received originally from Dr. Olympio da Fonseca, Rio de Janeiro, and cited by Thom and Raper (1945) as the basis for a new species in the *A. niger* group to include forms with large black heads, primary sterigmata of intermediate length (*ca.* 20–45μ), and large coarsely roughened conidia, mostly 6.5–8.0μ in diameter. This culture is of considerable economic interest since it produces either citric or gluconic acid in good yield, depending upon the conditions under which it is cultivated.

Tan: Induced mutation from *Stock* produced by irradiating conidia for one hour with monochromatic ultraviolet light, wavelength 2650 Å (Hollaender, Raper and Coghill, 1945). The mutant is characterized by conidial heads in light

to dull tan shades near cinnamon-buff to chamois (Ridgway, Pls. XXIX and XXX). Conidia are borne in comparatively short chains to form small, compact, globose heads. Dimensions of conidiophores, vesicles, primary and secondary sterigmata, and conidia in this mutant, and in most of the others examined, essentially duplicate the stock culture. The mutant grows at the same rate as the parent (Fig. 3A).

Pink: Induced mutation from *Tan*, characterized by compact, globose heads having short chains of conidia in light pink shades near salmon to flesh color (Ridgway, Pl. XIV). Growth on Czapek agar is somewhat restricted.

Bronze: Induced mutation from *Tan*, characterized by heads in dull orange-yellow shades near zinc orange to tawny (Ridgway, Pl. XV) with conidial chains often longer than in the tan mutant from which it is derived. Growth is satisfactory on all media.

Brown: Induced mutation from *Stock*, characterized by comparatively large conidial heads in brown shades near cinnamon-brown to mummy brown (Ridgway, Pl. XV). Growth is promptly initiated and proceeds rapidly on various natural substrata and on steep agar, but abundant sporulation is often limited to the central colony area. The mutant suffers from a biotin deficiency[2] which nearly precludes spore germination on unenriched Czapek agar, but growth proceeds in a normal manner once a microcolony has become established.

Orange: Induced mutation from *Stock*, characterized by fairly compact conidial heads in orange-yellow shades near yellow ocher (Ridgway, Pl. XV). Growth and sporulation are essentially normal on all substrata.

Fuzzy: Induced mutation from *Orange*. This bright yellow mutant, near lemon chrome (Ridgway, Pl. IV), is marked by very atypical heads of the *Cladosarum* type (Yuill and Yuill, 1938), wherein the secondary sterigmata fail to cut off normal conidia, but continue to proliferate, producing chains of pyriform vegetative elements. Growth is satisfactory on all substrata. The mutant is very short-lived, necessitating re-transfer at monthly intervals. Its intense pigmentation results from a rich deposit in the vesicles (Fig. 4E) and primary sterigmata of some crystalline coloring matter amounting in some cases to 35% of the dry weight of the fungus (Dr. F. H. Stodola, personal communication).

Honey. Induced mutation from *Orange*. Conidial heads are in chamois to honey yellow shades (Ridgway, Pl. XXX), globose, compact, commonly borne on long conidiophores. Colonies grow rapidly on steep agar, producing abortive conidial structures and sterile conidiophores abundantly in broad marginal areas to form a pattern of growth suggestive of *Brown* on the same substratum.

Thin: Induced mutation from *Stock*, characterized by its inability to utilize nitrate nitrogen, hence grows very sparingly and sporulates very lightly on Czapek agar. It grows normally and sporulates abundantly on natural and enriched substrata (*e.g.* steep agar) and in the presence of ammonium or amino nitrogen, producing colonies indistinguishable from *Stock* (NRRL 67).

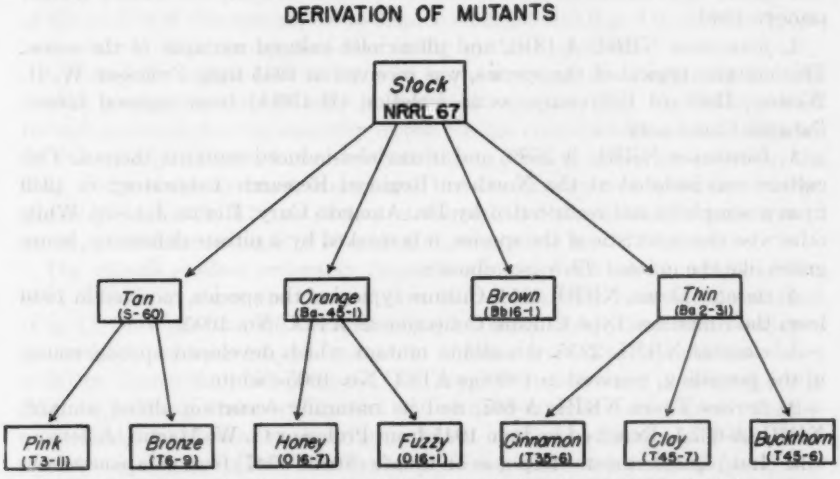
Cinnamon: Induced mutation from *Thin*. This mutant retains the thin growth habit of its immediate parent on Czapek agar, and additionally produces com-

[2] Determined by Dr. C. E. Harrold, California Institute of Technology.

pact conidial heads in shades of cinnamon (Ridgway, Pl. XXIX). In the presence of ammonium or amino nitrogen, as on natural and enriched substrata, growth is excellent and sporulation profuse.

Buckthorn: Induced mutation from *Thin*. This mutant differs from the preceding in being somewhat longer-stalked and in producing conidial heads in orange to light brown shades near buckthorn brown (Ridgway, Pl. XV). The nitrate deficiency is retained.

Clay: A third mutation from *Thin* which produces conidial heads near clay color (Ridgway, P., XXIX). Superficially, it closely resembles *Buckthorn* but was isolated from another site, hence from a different mutated conidium.



DIAG. 1. Origins and relationships of ultraviolet-induced mutations of *A. fonsecaeus* Thom and Raper, NRRL 67, included in the present study. Mutant characteristics are given in the text.

The cultures described above represent selected mutations with which we have worked most intensively and constitute only a small fraction of the mutations isolated during this study. Their origins and relationships are shown diagrammatically in Diag. 1. The designations applied to the mutants represent descriptive names assigned for convenience in the laboratory and are not intended to imply any taxonomic status.

The mutant *Thin* was originally observed as an area of thin submerged growth bearing widely scattered conidial heads. Without exception, the color mutants have been first identified as limited groups of conidial heads possessing some distinctive characteristic, and isolations made from such structures have yielded stable mutants after one or more transfers. Pure mutant colonies have not been observed in dilution platings of irradiated conidia. Whereas the development of such would be theoretically possible, their absence can be readily appreciated since the conidia of *A. fonsecaeus* are multinucleate (p. 7). To obtain a pure

mutant colony in platings of irradiated conidia would require that a mutation occur in a single nucleus of a conidium while all other nuclei are killed without destroying cell viability. A more probable explanation of their origin, and one which fits observed behavior, is that a single nucleus in a conidium is mutated while at least some, and possibly all, of the remainder are unaffected. In ensuing colony development a mutated nucleus becomes walled off in a single cell, which then proliferates to produce a limited area of mutant mycelium that gives rise to the observed mutant conidial structures.

In addition to *A. fonsecaeus* NRRL 67 and the described mutants thereof, other strains and species examined in this investigation included:

A. fonsecaeus NRRL 591, from Dr. Dorival M. Cardozo, Sao Paulo, Brazil, prior to 1940.

A. fonsecaeus NRRL A-1310, and ultraviolet-induced mutants of the same. This culture, typical of the species, was received in 1945 from Professor W. H. Weston, Harvard University, as an isolation (H-4B3A) from exposed fabric, Panama Canal Zone.

A. fonsecaeus NRRL A-2589, and ultraviolet-induced mutants thereof. This culture was isolated at the Northern Regional Research Laboratory in 1949 from a sample of soil contributed by Dr. Amando Cury, Rio de Janeiro. While otherwise characteristic of the species, it is marked by a nitrate deficiency, hence grows like the mutant *Thin* (see above).

A. clavatus Desm. NRRL 2254. Culture typical of the species, received in 1946 from the American Type Culture Collection as ATCC No. 1005.

A. clavatus NRRL 2255. An albino mutant which developed spontaneously in the preceding, received in 1946 as ATCC No. 1005-"white."

A. terreus Thom NRRL A-667, and its naturally occurring albino mutant, NRRL A-667A. Received in June 1945 from Professor G. W. Martin, Jeffersonville (Ind.) Quartermaster Depot as an isolate (Strain J347) from a Japanese belt, from vicinity of Hollandia, New Guinea.

A. fumigatus Fres. NRRL A-1173, and its naturally occurring mutant, NRRL A-1173A. Received in Nov. 1945 from Dr. E. R. Kooi, Argo, Illinois, as an isolate (Strain M-1007E) from filter paper.

Strain Intermixtures in Aspergillus Fonsecaeus NRRL 67 and Its Mutants

Stock × *Tan*

Preliminary investigations revealed that when the tan-spored mutant (*Tan*) and the black-spored parent, NRRL 67 (*Stock*), were planted side by side, scattered conidial heads of intermediate form and color regularly developed along the interface between tan and black colony areas. Subcultures established by mass inoculation (*i.e.* multiple conidia) from such heads characteristically developed as mixed colonies containing typical black, typical tan, and a variety of intermediate-type heads, or blends. When replanted, typical black heads yielded only black colonies of the original parent-type, and typical tan heads yielded only

tan colonies characteristic of the mutant, whereas intermediate heads gave rise to mixed colonies showing all three head-types in varying proportions. The situation seemed to parallel exactly that described by Gossop and the Yuills (1940).

However, when dilution plate cultures were made with conidia taken from heads of intermediate pigmentation, a strikingly different development occurred. Many pure tan and some pure black colonies were observed while, in addition, varying numbers of mixed black and tan colonies usually developed. Unlike the results of Gossop *et al.*, isolation of single conidia in repeated experiments verified the fact that mixed colonies were developing from individual spores. A reasonable explanation seemed to demand that the conidia of *A. fonsecaeus* NRRL 37, and its mutant, were multinucleate, or at least binucleate. The large dimensions of the conidia of this species, 6.0 to 8.5μ, in contrast to 3.0 to 4.0μ in most members of the *A. niger* group, lent plausibility to this hypothesis.

Cytological studies established with certainty that the sterigmata and the conidia arising from them were in fact multinucleate (Fig. 1). These studies further revealed that the majority of the conidia contained about 5 or 6 nuclei. It was usually difficult to demonstrate the number of nuclei contained in the long, wedge-shaped primary sterigmata, but the number was found to be large, probably 15 to 20. The secondary sterigmata contained smaller numbers of nuclei, seldom exceeding 8 or 10 and often numbering less.

The conidia swelled noticeably in germination and the nuclei usually underwent division at about the time, or even before, the germ tube became evident (Fig. 1). Once germinated, the sporeling grew fairly rapidly and soon developed into a freely branching hyphal network. Hyphae from other conidia interlaced with the elements of this network, and hyphal anastomoses developed freely (Fig. 1). In areas where both parent and mutant mycelia were present, anastomoses and concomitant nuclear intermixture of the tan mutant and the black parent was commonplace. Conidiophores arising from such heterokaryotic mycelial elements naturally contained both "black" and "tan" nuclei, and these appeared successively in the enlarging vesicle, in the large primary sterigmata, and in the smaller and very numerous secondary sterigmata which represent the ultimate conidiiferous cells. By the repeated division and migration of nuclei from such reproductive cells, the multinucleate and heterokaryotic condition was carried into the chains of developing conidia (Fig. 1). The intermediate type heads, or blends, observed in mixtures of *Stock* × *Tan* appeared to be entirely homogeneous when viewed with low or high magnifications, and showed no evidence of striation or mottling as reported by the English workers in many of the heterokaryons studied by them. Such apparent homogeneity could not be wholly explained by the heterokaryotic character of the constituent conidia since seldom more than 50% of those from the most thoroughly mixed heads produced mixed colonies upon cultural analysis. Adopting the terminology introduced by Pontecorvo (1946) the mixed heads would be designated as "nonautonomous", wherein pigmentation is determined by the heterokaryotic conidiophore rather than the nucleus (or nuclei) segregated in each conidium.

Whereas such heads of intermediate character regularly developed along the

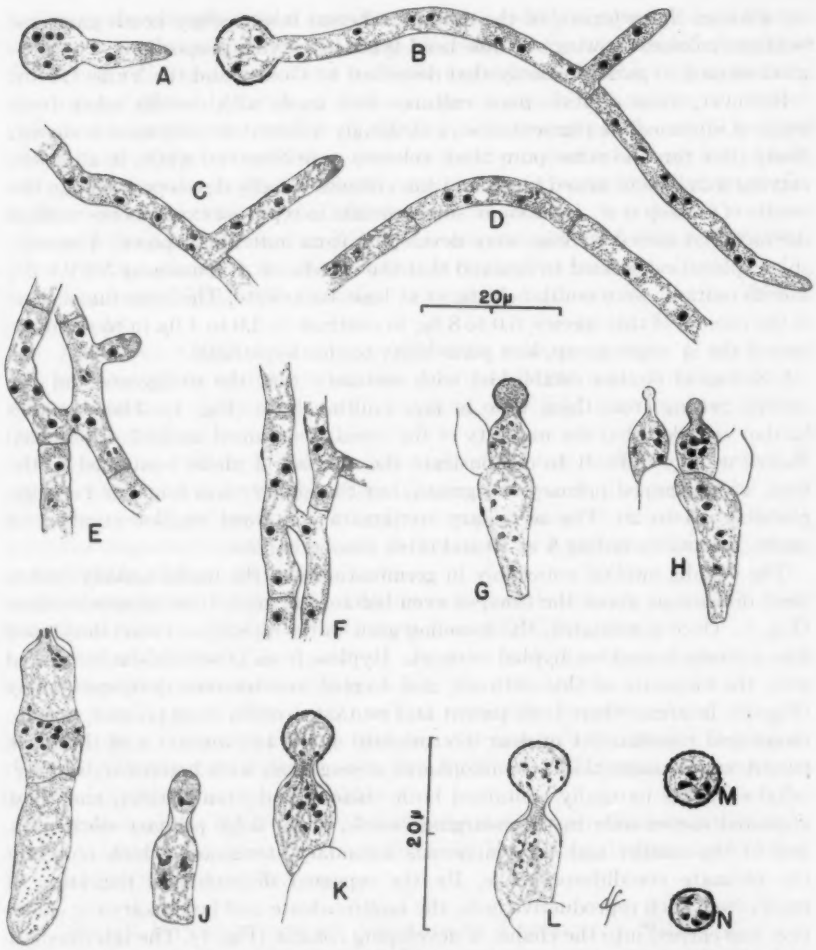


FIG. 1. Camera lucida drawings of stained preparations of *A. fonsecaeus*, NRRL 67. A, germinating conidium; B, elongating germinal hypha showing nuclei and the formation of an initial branch; C and D, limited portions of vigorously growing mycelium showing nuclei in various stages of division; E and F, anastomoses between adjacent hyphae; G and H, detail of primary and secondary sterigmata and developing conidia; I, J, and K, selected structures showing stages in spore formation, including the migration of nuclei into the developing conidia; M and N, mature conidia.

juncture of black and tan colony areas when these were planted side by side (Fig. 2A and B), much greater numbers were produced in the mixed colonies which resulted from single heterokaryotic conidia (Fig. 2C and D). This undoubtedly resulted from increased anastomoses giving rise to a more completely heterokaryotic vegetative mycelium in the latter case.

So-called intermediate-type heads varied greatly in size, color, and pattern, and covered the entire range from large, black, loosely-dissected heads typical of the parent to the compact, comparatively small, tan-colored heads of the mutant. While in no sense exact, some general relationship seemed to exist between the overall appearance of a mixed head and the relative numbers of the

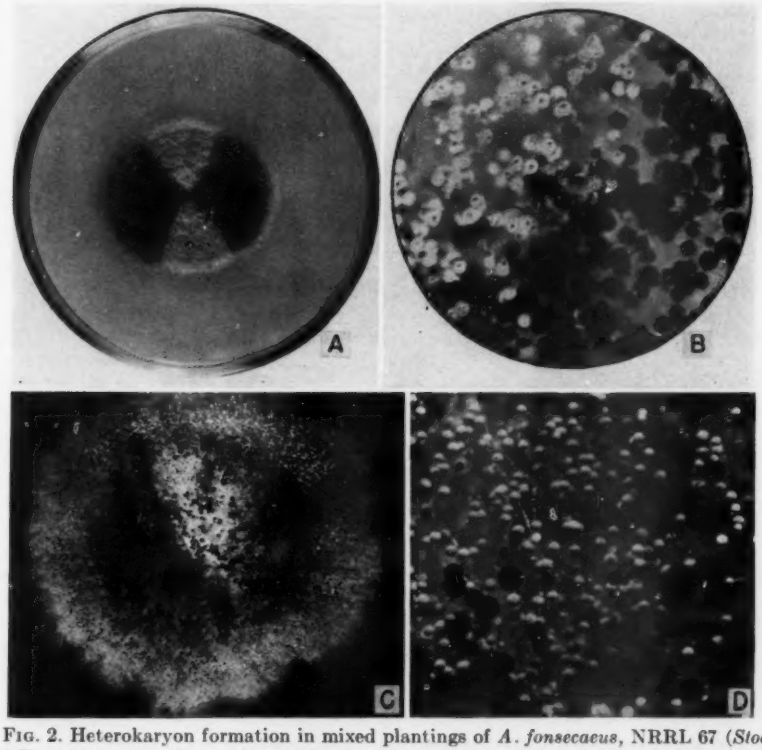
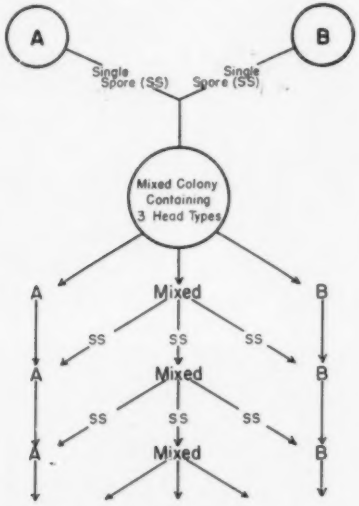


FIG. 2. Heterokaryon formation in mixed plantings of *A. fonsecaeus*, NRRL 67 (*Stock*) and *Tan*. A, pattern of growth resulting from implanting conidia of the opposed strains at diagonally opposite corners of an imaginary square (*ca.*1 cm.), on Czapek agar after 7 days at room temperature; B, limited portion of parent-mutant interface enlarged to show presence of a heterokaryotic head (arrow), × 9.5; C, colony developed from a single heterokaryotic conidium, on Czapek agar after 10 days at room temperature; D, limited field from central area of same colony showing black, tan, and heterokaryotic heads (arrows), × 8.5.

different types of colonies that developed from its conidia. When taken from an area of heavy intermixture, small, compact heads in light brown shades usually yielded only pure tan colonies. Chocolate-colored, medium-sized, loose-textured heads yielded colonies of all three types, but with tan colonies predominating. In the cultural analysis of one series of 10 such heads made by dilution plate techniques, an average of 60% of all colonies developed as pure tan, 34.6% as mixed, and 5.4% as pure blacks. Only from heterokaryotic heads which super-

ficially appeared "black" did we succeed in securing a population where a majority of the colonies were mixed and where pure blacks outnumbered pure tans.

Gossop *et al.* (1940) demonstrated heterokaryotic (HC) heads to yield mixed colonies composed of both HC heads and heads typical of each mutant, or mutant and parent strain, entering into the mixture. In *A. fonsecaeus* the same situation applies, *but to individual conidia as well as to conidial heads.* The relationship is illustrated in the accompanying diagram (Diag. 2).



DIAG. 2. Schematic diagram showing the origin of a heterokaryotic colony from the pairing of two mutants (or parent and mutant), and the kinds of colonies that develop from the three head types produced therein, as determined by single spore analyses.

Stock × *Orange, Pink, Bronze, and Brown*

Interstrain mixtures of *Stock* × *Orange* showed a pattern of behavior like the preceding. At colony interfaces, heterokaryotic heads of intermediate coloration (blends) were developed, and upon cultural analysis individual conidia from these yielded pure orange, pure black, and mixed orange and black colonies.

Intermixtures of *Stock* × *Bronze* or *Pink* have yielded inconclusive results, and doubtfully produce heterokaryotic heads and conidia. In each case conidial heads intermediate in color between the black parent and the more lightly pigmented mutant have been observed, and records of some preliminary trials indicated the formation of occasional heterokaryotic structures. More recent tests have failed to verify such conidial heterogeneity, although like structures which superficially resembled blends were observed.

Perhaps due to insufficient differences in conidial colors, heads suggesting presumptive heterokaryons were not observed in mixing plantings of *Stock* × *Brown*.

Orange × *Brown*

When the orange and brown mutants were planted opposite one another, a striking development occurred. Along colony interfaces of the two mutants a substantial number of very large, deep black conidial heads appeared which

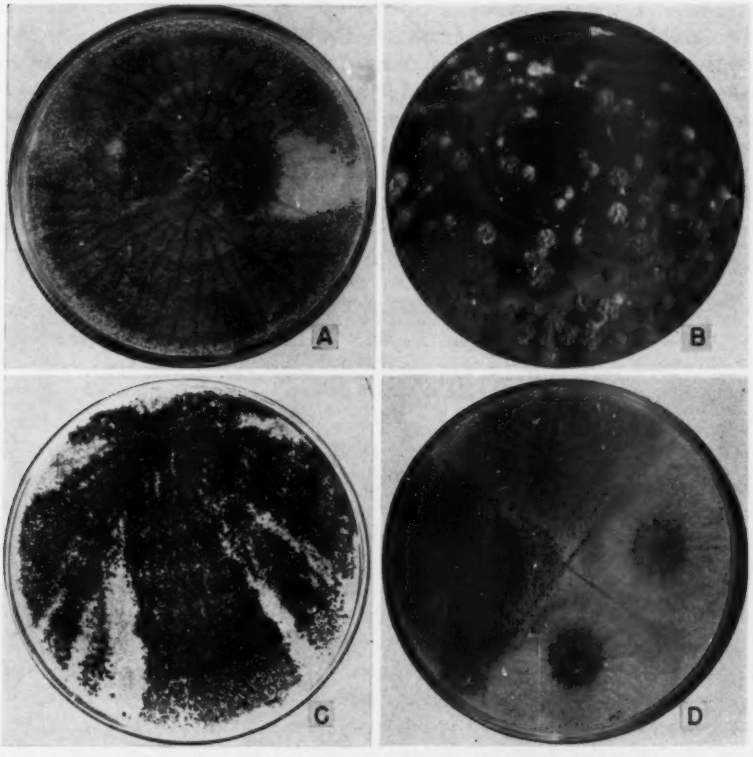


FIG. 3. Heterokaryon formation in mixed plantings of *Orange* × *Brown*. A, ten-day old culture on steep agar showing black heterokaryotic heads at *Orange* (spreading)-*Brown* (restricted) interface; B, limited area of the same to show large black heterokaryotic heads, brown mutant above, orange below, × 9.5; C, colony developed from a single heterokaryotic conidium wherein strongly heterokaryotic growth predominates as shown by areas of black conidial structures; D, plate containing colonies developed from individual conidia isolated from a single chain of spores removed from a strongly heterokaryotic head.

strongly suggested the typical fruiting structures of *Stock* (Fig. 3A and B), the immediate parent of both mutants. Upon cultural analysis, conidia from such black heads yielded pure orange, pure brown, and mixed colonies, the latter being composed of varying proportions of typical orange, typical brown, and large black conidial heads. Subcultures made from typical orange and brown heads remained pure and characteristic of the two mutants, respectively. Sub-

cultures made from black heads yielded some pure colonies of the two mutants and additional mixed colonies in which the three distinctive head types reappeared. This relationship is shown in Diag. 2.

Basically, the situation in *Orange* × *Brown* mixtures is like that resulting from mixed plantings of either *Tan* or *Orange* × *Stock*, except that the heterokaryotic heads appear in the true form and color of the wild type rather than as blends between black and the lighter pigmentation of the mutant. It seems reasonable to assume that the distinctive coloration of each mutant results from the loss of some factor (or factors) necessary for the elaboration of black pigment; and when the two types of mutant nuclei enter a common fruiting structure a sufficient complement of essential factors is restored for the development of apparently typical black heads.

TABLE I

Colonies developed in dilution plates from ten heterokaryotic heads in Orange × Brown mixtures

Spore suspensions plated at 1:100 dilution on steep agar

HEAD NO.	NUMBERS (PERCENTAGES) AND TYPES OF COLONIES		
	Orange	Mixed	Brown
Mutant Interface—Large, black head			
1	7 (70%)	1 (10%)	2 (20%)
2	15 (56%)	3 (11%)	9 (33%)
3	24 (86%)	1 (3%)	3 (11%)
4	8 (21%)	11 (29%)	19 (50%)
5	11 (25.5%)	14 (32.5%)	18 (42%)
6	6 (14%)	21 (49%)	16 (37%)
Heterokaryotic Sectors—Small, black head			
7	2 (22%)	5 (56%)	2 (22%)
8	1 (3%)	12 (37%)	19 (60%)
9	5 (28%)	10 (55%)	3 (17%)
10	5 (12%)	16 (76%)	5 (12%)

Here, as in the heterokaryotic structures of intermediate color produced in mixtures of *Stock* × *Tan*, the mixed heads apparently result from nonautonomous nuclear action (Pontecorvo, 1946). Superficially and microscopically, these conidial structures generally appear to be homogeneous throughout, and we have not seen striate or mottled heads that would suggest differences among the spore chains that comprise them. The relative proportions of conidia which develop pure orange, pure brown, and mixed colonies vary widely in different and equally black heterokaryotic structures. Additionally, no correlation between such populations and the size of the conidial heads has been observed, although it may be noted that black heads developed at mutant colony interfaces are regularly larger than the heads which are formed within a colony of *Stock* or in the interior of a black, and hence strongly heterokaryotic, sector of a mixed colony (Fig. 3C).

These relationships are shown in the accompanying tabulation based upon dilution platings of the conidia from ten black heads. The general characteristics and sources of the heads are given, and the ratios of resulting colony types are expressed as numbers of colonies (1:100 dilution) and as percentages of the total population.

As with types of colonies which develop from the conidia of heterokaryotic heads, the relative proportions of orange, brown and black (mixed) heads which develop in colonies arising from individual heterokaryotic conidia vary markedly. These range from (1) colonies wherein one mutant (usually *Orange*) predominates, while the other mutant and the heterokaryotic areas, essentially black, appear as narrow sectors or localized areas, to (2) colonies wherein most of the growth is heterokaryotic (Fig. 3C) and one or both mutants appear as narrow sectors, or as localized areas. In no case, either of colonies developing from different conidia, or of head types in colonies developing from single spores, have we observed a ratio which suggests a pattern of predictable inheritance. As one might expect, the distribution of spores within conidial heads, and that of nuclei within individual conidia, is obviously one of chance in structures which arise from heterokaryotic mycelia. We assume, however, that the populations produced (heads or conidia) reflect with fair accuracy the relative numbers of the two types of mutant nuclei contained in the heterokaryon (colony or fruiting structure).

In attempting to analyze the nuclear constitution of heterokaryotic heads and in conidia in mixtures of *Orange* × *Brown*, it became of interest to determine whether or not all of the conidia developed from a single secondary sterigma were of similar nuclear constitution. Chains of conidia were separated by subjecting single black heads, removed and held above sterile agar plates by jewelers' forceps, to short blasts of air from a finely drawn pipette. Agar blocks bearing isolated chains of conidia were then removed to fresh agar plates and the conidia in the chains were drawn apart by very thin glass needles, allowed to germinate, and isolated by the method already cited (p. 2). Fourteen spore chains, containing from 7 to 23 conidia each, were selected from two black heads for analysis. Eight chains yielded only pure colonies of the orange mutant; two of the remaining six yielded mixed colonies only; whereas the remaining four produced mixed colonies together with colonies of either one or both mutants. In a subsequent experiment, conidia from single spore chains of a heterokaryotic (black) head from *Tan* × *Orange* similarly yielded colonies of three types, namely, pure tan, pure orange and mixed. The fact that spores of three types are found in one chain, hence developed from a single sterigmatic cell, clearly demonstrates that the divisions of the nuclei in such a cell are independent of each other and that the complement of daughter nuclei which enters any given conidium is a matter of chance. Figure 3D illustrates a case where each of three conidial types, orange, brown and mixed, occurred within a group of four spores from a single chain.

Additional Intermixtures of Color Mutants

Following the discovery that *Orange* × *Brown* yielded large, black heterokaryotic heads, other selected color mutants of NRRL 67 were planted in juxta-

position to *Orange* and to each other to determine whether they produced mixed heads of a similar type. The results are summarized in Diag. 3. When *Orange* was planted adjacent to *Tan*, *Pink* or *Bronze*, occasional large black heads developed at colony interfaces, and these, upon cultural analysis, yielded pure colonies of the two mutants and mixed colonies comprised of pure mutant (s) and abundant mixed black heads. Hence, results duplicated those obtained with

STRAINS INTERMIXED	Stock	Tan	Pink	Bronze	Brown	Orange	Fuzzy	Thin
NRRL 67 (Stock): Conidial heads large, black; conidia large, ca. 6.5-8.0μ diam.								
Tan: Conidial heads compact, in light to dull tan shades	◐							
Pink: Conidial heads compact, globose, in light pink to flesh shades	◉	○						
Bronze: Conidial heads medium-sized, in shades near bronze	◉	○	○					
Brown: Conidial heads medium-sized, in light to dull brown shades. Biotin deficiency	□	⊗	⊗	⊗				
Orange: Conidial heads compact, in yellow-orange shades	◐	●	●	●	●			
Fuzzy: "Conidial" heads Cladosarum-like, bright yellow	○	●	●	●	●	○		
Thin: Conidial heads large, black, scantily produced on Czapek's agar. Nitrate deficiency	□	◐	◐	◐	□	◐	◐	

◐ Blends: heterokaryotic heads and conidia.

● Black heterokaryons: heads and conidia.

◉ Blends(?): heterokaryosis rare or doubtful. (See text)

⊗ Black heads: presumptive heterokaryons yield pure Brown. (See text)

○ Heterokaryons not observed.

□ Heterokaryons not observed: inadequate pigment differentiation.

DIAG. 3. Varied patterns of behavior observed when the parent culture, *A. fonsecaeus* NRRL 67 (*Stock*) and selected mutations derived from it are paired in all possible combinations. (See discussions in text.)

Orange × *Brown* except for the less frequent appearance of heterokaryotic heads in primary pairings.

When paired among themselves, *Tan*, *Pink* and *Bronze* exhibited no obvious evidence of intermixture, and heads selected from their colony interfaces developed only pure colonies of one or the other mutant in dilution platings. Such behavior might be anticipated in part. *Pink* and *Bronze* were produced by irradiating conidia of *Tan*, a mutant itself characterized by lightly pigmented spores, and one, we presume, from which some factor essential for the production of black pigment had already been lost (see p. 3). Recombining this mutant with

one of its progeny, or the progeny between themselves, could hardly be expected to restore the wild type pigmentation. As a matter of fact, it seems probable that other unknown losses accompanying altered pigmentation probably precluded heterokaryon formation, otherwise we might have expected intermixtures to be manifest as blends. Even these have not been demonstrated.

In combinations of *Brown* × *Tan*, *Pink* or *Bronze* large black (and presumptively heterokaryotic) heads developed at the mutant interfaces. Upon cultural analysis, however, such heads, without exception, yielded only pure colonies of *Brown*. Two significant developments may be noted in Diag. 3. *Orange* obviously possesses an exceptional facility for hyphal anastomosis and the subsequent formation of true heterokaryotic fruiting structures. On the other hand, *Pink*, *Bronze* or *Tan* in combinations with *Brown* evidently stimulates this mutant to produce large black heads at colony interfaces which simulate true heterokaryons but which in reality represent pure *Brown*. Do nuclei of these three mutants enter the mycelium of *Brown* but in numbers so small that they fail to reach the conidia? Do they in some manner stimulate *Brown* to produce black conidial heads in the absence of nuclear migration? No satisfactory explanation for this behavior can yet be advanced. Phenomena which are apparently comparable to this, but even more striking, were subsequently encountered in combinations of *Brown* or *Orange* with selected color mutants of *Thin*. Additional attention is given to this matter in our consideration of such pairings (see p. 19).

Intermixtures with Thin

Thin, the nitrate deficient mutant of *Stock*, was planted adjacent to *Tan*, *Pink*, *Bronze*, *Orange* and *Brown*. In combinations of *Thin* vs. *Tan*, *Bronze*, and *Orange* conidial heads of intermediate pigmentation (blends) were observed at mutant interfaces. Upon cultural analyses, these proved to be heterokaryotic, yielding pure colonies of each mutant and mixed colonies comprised of pure mutant(s) and mixed heads of intermediate color (Diag. 3).

As one might expect, since *Thin* carried the normal black pigmentation of the parent culture (NRRL 67), heterokaryotic heads formed in mixed plantings with it were colored in shades intermediate between the opposed mutants. In other words, there was a dilution of black by the lighter pigmented mutant.

Heterokaryotic heads were doubtfully formed in pairings of *Thin* × *Pink* (Diag. 3).

No evident intermixture occurred in *Brown* × *Thin*, and conidia from black heads taken from the interface of mutant colonies yielded only pure colonies of *Thin*. It should be noted, however, that in this case, as already stated for *Stock* × *Brown*, differences in mutant pigmentations were not sufficiently marked to provide even presumptive evidence of heterokaryons.

Intermixtures with Secondary Color Mutations of Orange

Intensely heterokaryotic cultures were obtained from mixed plantings of *Brown* and secondary ultraviolet induced mutations of *Orange*. A lightly pigmented strain with near normal fruiting structures designated as *Honey* is repre-

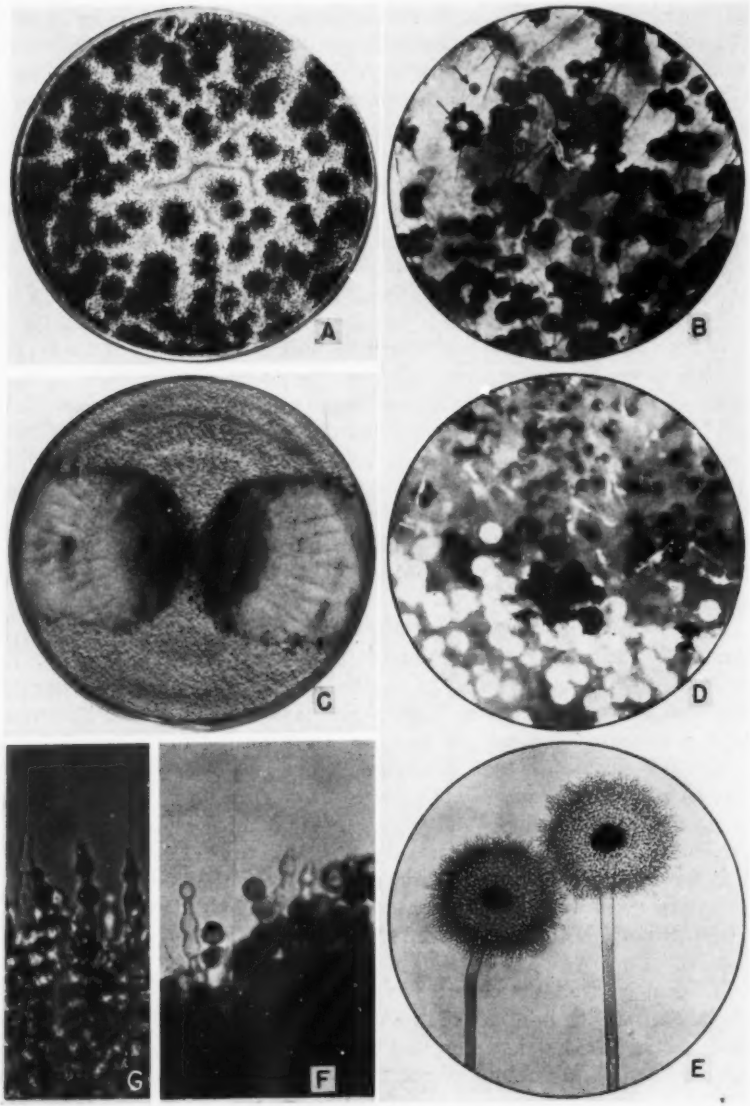


FIG. 4

sentative of these, and results obtained with it will be summarized. The original pairings of *Honey* × *Brown* were made in the usual manner, and large black heterokaryotic heads developed abundantly at colony interfaces. When plated, conidia from certain of these heads yielded colonies which were predominantly black with typical heads of each mutant appearing only in small scattered clumps. Recultivation of conidia from heads situated within the blackest areas yielded cultures which superficially appeared "all black" (Fig. 4A), but which under low power revealed occasional heads of each mutant (Fig. 4B), often as isolated structures in an otherwise dense stand of black heads. This intensely heterokaryotic condition has been maintained through subsequent recultivations from the black heads and we believe that an essentially stable heterokaryon has been established.

Pairings of *Fuzzy* × *Brown* yielded heterokaryotic conidial structures of particular interest. In previous tests we had worked only with mutants characterized by altered pigmentations or nutrient deficiencies. In this case, a marked structural modification was introduced, since the secondary sterigmata of *Fuzzy* produce rows of adherent, pyriform vegetative cells (Fig. 4E, F, and G) rather than chains of well-separated, globose conidia. Large, black heterokaryotic heads were produced in limited numbers along the interfaces of mutant colonies (Fig. 4C and D). Superficially, these resembled the wild type and were, in fact, oftentimes indistinguishable from the mixed heads regularly produced in pairings of *Brown* × *Orange*, the latter being the immediate parent of *Fuzzy*. Microscopically, the heterokaryotic heads were seen to consist of apparently normal sterigmata bearing chains of dark globose conidia and lesser numbers of abnormal sterigmata bearing rows of adherent, pyriform cells (Fig. 4F and G) characteristic of *Fuzzy*.

Upon cultural analysis, some large black, and presumptively heterokaryotic heads, yielded pure brown colonies only. In other cases, three types of colonies were observed to develop from black heads which were apparently similar to these. The greatest degree of intermixture was obtained generally from heterokaryotic structures which showed a limited development of "fuzzy" cellular elements projecting beyond the black spores (Fig. 4F, G). The pyriform elements and their supporting abnormal sterigmata are considered to contain only nuclei

FIG. 4. Heterokaryon formation in mixed plantings of *Brown* × two secondary mutations of *Orange*. A, strongly heterokaryotic culture of *Brown* × *Honey*, wherein the two contributory mutants appear individually only as widely scattered conidial heads; B, an area of the same enlarged to show three "pure" heads of *Honey* (arrows) in a field of otherwise black heterokaryotic heads, × 9.5; C, *Brown* × *Fuzzy* planted in conventional fashion on steep agar (10 days at room temperature) showing numerous black heterokaryotic heads at *Brown* (right and left)—*Fuzzy* (top and bottom) interfaces; D, an area of the same somewhat enlarged to show a typical, large heterokaryon, × 9.5; E, two conidial heads of *Fuzzy* showing their characteristic *Cladosarum*-like form and structure, the black central areas resulting from the rich deposition of an unknown yellow pigment (see p. 4), × 70; F, detail of abortive "sterigmata" characteristic of *Fuzzy*, × 475; G, detail of conidial head surface in a heterokaryotic structure, note the non-sporulating sterigmata (*Fuzzy*) projecting beyond a layer of well-formed black conidia which are borne on heterokaryotic sterigmata of normal pattern, × 475.

of the fuzzy mutant, whereas the well-formed black spores undoubtedly contain nuclei of both types, or of the *Brown* mutant only. Large black heads which yielded populations of pure brown colonies only were commonly produced and these were regarded as probably comparable to the large black heads observed in paired cultures of *Brown* × *Tan*, *Pink* or *Bronze* (p. 15). Mixed (black) heads with abundant heterokaryotic conidia are not infrequently produced, however, and colonies resulting from such spores showed a degree of admixture quite comparable to those similarly obtained from heterokaryotic conidia of *Orange* × *Brown*.

Interestingly enough, the optimal method for maintaining the mutant *Fuzzy* is to lyophilize heterokaryotic conidia in which it is contained. The pyriform vegetative cells of this form cannot be lyophilized successfully, and the mutant alone in agar slants and plates dies out within a very few weeks. As a matter of fact, the mutant has been twice recovered *via* the heterokaryon route, in the course of our study, when conventional cultures of the pure mutant had been lost.

Although derived from *Orange*, which mixes readily with *Tan*, *Pink* and *Bronze*, *Fuzzy* produces heterokaryotic heads sparingly when planted in apposition to these color mutants. Upon conidial analysis in dilution plates, however, such black heads show a thorough intermixture of the opposed mutants (Diag. 3). Strongly heterokaryotic cultures thus established can be readily maintained.

Intermixtures with Color Mutations of Thin

Conidia of *Thin*, the nitrate-deficient mutant of NRRL 67, were irradiated with ultraviolet light and a number of secondary mutations were obtained wherein differences in conidial pigmentation were superimposed on the already existing nutrient deficiency. It seemed of interest to plant these in apposition to the well-defined color mutations already investigated in the somewhat optimistic hope that some type of recombination of characters might occur in developing heterokaryons. The mutants were paired in the conventional manner employing steep agar, and as mixed point inoculations on Czapek and high-sugar agars. Demonstrable heterokaryotic fructifications developed more consistently in the latter type of culture. The results of such pairings are summarized in Diag. 4, where paired mutants are recorded as positive, irrespective of the conditions under which heterokaryons developed.

Whereas no evidence of character recombinations was observed in any instance, a number of significant facts were revealed. The color mutations of *Thin* formed heterokaryons in all but 4 of 18 combinations tested (Diag. 4). Behaviors differed markedly, and certain of the intermixtures merit consideration.

In some cases, *e.g. Cinnamon* × *Pink* or *Bronze*, heterokaryotic heads developed only occasionally along mutant colony interfaces on steep agar and as scattered structures throughout the colonies developing from mixed inocula on Czapek and high-sugar agars. Heterokaryotic heads in these mixtures, and in combinations of *Cinnamon* × *Tan*, the mutant from which *Pink* and *Bronze* were derived, were not black like the wild type but appeared in various dull to

dark grayish brown shades. The same phenomenon was observed in *Clay* × *Pink*.

In other cases, *e.g. Clay* × *Brown*, scattered black, heterokaryotic heads developed in the dense central areas of colonies resulting from mixed point inoculations on Czapek and high-sugar agars. When paired on steep agar in the conventional way, the same strains produced conspicuous bands of large black conidial heads along the colony interfaces (Fig. 5A and B). Such heads were presumed to be strongly heterokaryotic. However, when dilution platings were made in repeated experiments, black heads of such origin yielded populations of pure *Brown* only (Fig. 5C). The explanation for this behavior is not known. Some possibilities are considered herewith.

MUTANTS	Tan	Pink	Bronze	Orange	Fuzzy	Brown
Cinnamon	⊗	⊗	⊗	●	●	●
Clay	○	⊗	○	●	●	●
Buckthorn	●	●	●	○	○	●

● Black heterokaryons: heads and conidia.
⊗ Gray-brown heterokaryons: heads and conidia.
○ Heterokaryons not observed.

DIAG. 4. Patterns of behavior observed in mixed plantings of three color mutations of *Thin* (nitrate deficient) and six selected color mutations of *Stock* previously investigated. (See discussions in text.)

Nuclei of the clay-colored mutant may have entered developing conidiophores that were predominantly *Brown* and, for some reason, never reached the level of the sterigmata where they could be cut off in conidia. Nevertheless, their presence (with accompanying cytoplasm) in the developing conidiophore, conceivably in very small numbers, may have contributed to the production of a uniformly colored fructification by nonautonomous nuclear action, albeit all the conidia subsequently formed apparently contained nuclei of a single kind rather than of two types as illustrated by Pontecorvo (1946), and, for that matter, as found in the vast majority of heterokaryons examined in this study.

Nuclei of *Clay* may actually have reached the level of the conidia, and, for some cause, spores containing such nuclear material may have failed to germinate. This, however, seems improbable since dilution platings of conidia from these presumptively heterokaryotic heads showed a percentage germination quite comparable with those observed in pure cultures of the two opposed mutant strains and in conidia from known heterokaryotic heads such as those produced in *Orange* × *Brown*.

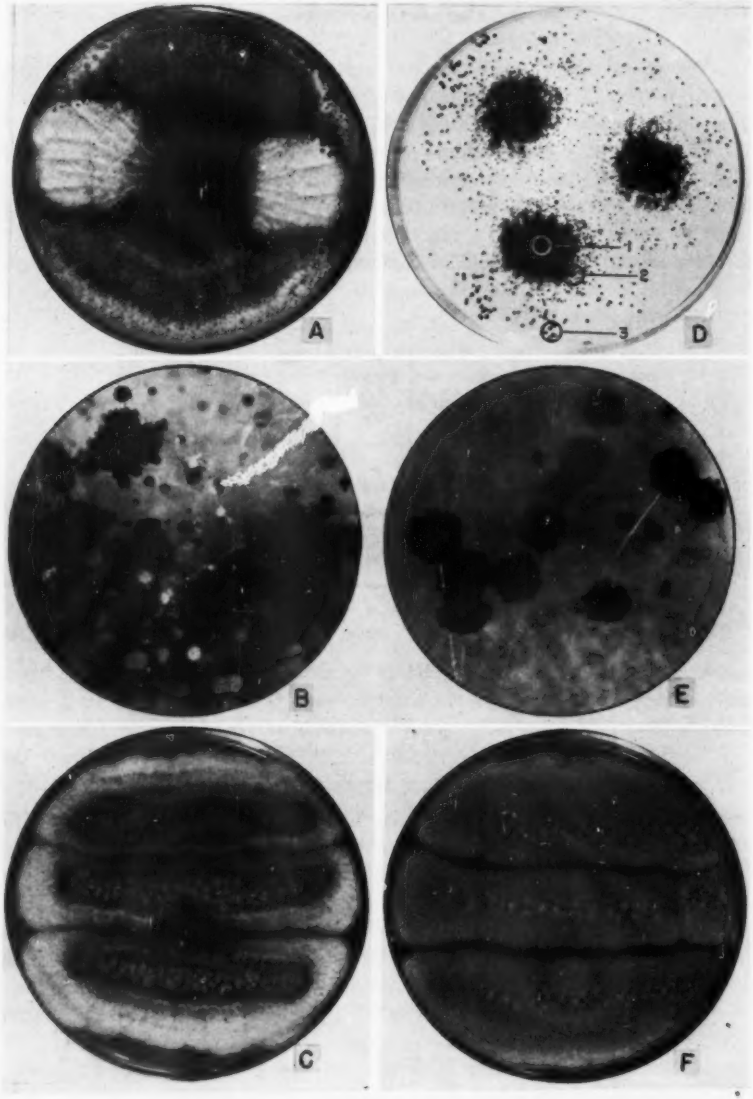


FIG. 5

The elaboration of a diffusible substance by *Clay* which prompted the production of large black conidial heads by *Brown* in the absence of any nuclear intermixture represents still another possibility. Attempts to demonstrate such a substance (by the use of permeable membranes, etc.) have failed thus far, but certain observations suggest its credibility. For example, when conidial heads of two lightly pigmented mutants, *e.g. Pink* and *Orange*, are contiguous, the area of physical contact approaches black, a phenomenon previously observed and illustrated by Gossop and co-workers (1940) in certain mutant intermixtures of *A. niger*. The area of such blackening is at most limited to a few hundred microns in width, but at the same time it occurs under conditions which preclude nuclear migration and intermixture.

Interesting and, we believe, significant information is provided by paired cultures of *Clay* × *Brown* grown from point inoculations on high-sugar agar (Fig. 5D). Centrally, these two mutants grow together as a compact, thoroughly intermixed, heavily sporulating colony containing some black heads which are strongly heterokaryotic (Fig. $5D_1$). Outward from this dense area, growth thins progressively and in the transitional zone other large black heads regularly occur (Fig. $5D_2$). In the marginal area of the plate vegetative growth is sparse and almost wholly submerged, and scattered long-stalked, almost black conidial heads are produced (Fig. $5D_3$ and E). Superficially the conidial heads of both the latter areas suggest strongly heterokaryotic structures. However, upon conidial analysis the first of these generally yields pure populations of *Brown*, whereas the latter yields only the thin-color mutant (Fig. 5F). The relationship between the two mutants appears to be a reciprocal one, for in one area of such a culture the presence of *Clay* apparently stimulates in *Brown* the production of black conidial structures, while in another area of the same plate *Brown* stimulates the thin color mutant to produce essentially black heads. Whereas nuclei and/or cytoplasm of *Brown* were not present in sufficient numbers or amount in marginal plate areas to alter appreciably the nitrate deficient growth pattern of *Clay*, the possible migration of such components from one mutant into the mycelium of the other is not excluded. As a matter of fact, heads shown to be heterokaryotic by dilution plate analysis have occasionally been demonstrated in the transitional zone between the dense central colony area and the wide thinly

FIG. 5. Responses observed in mixed plantings of *Brown* × *Clay*, a color mutation of *Thin*. A, plate culture of *Brown* (restricted) × *Clay* (spreading) planted in conventional fashion, showing dense bands of presumptive heterokaryons at mutant-mutant interfaces, 10 days on steep agar at room temperature; B, limited portion of such an interface to show conidial structures substantially enlarged, × 9.5; C, streak culture inoculated with a spore suspension from a presumptive heterokaryon which characteristically yielded *Brown* as a pure culture; D, culture of *Brown* × *Clay* wherein the two mutants were paired as point inoculations on high-sugar agar: at colony centers (D_1) black heads commonly represent true heterokaryons, at edge of dense growth (D_2) they usually yield *Brown* in pure culture, whereas in marginal colony areas (D_3) they produce only pure colonies of *Clay*; E, enlarged view of black heads from marginal colony area, × 9.5; F, pure culture of *Clay* developed from suspension of spores from heads as seen in preceding. (See discussion in text.)

growing marginal zone. In a single instance a heterokaryotic head was encountered well within the latter area. In these cases prior hyphal anastomoses and nuclear migrations are incontrovertible. Perhaps they regularly occur in varying degree throughout the entire plate, oftentimes only at a level sufficient to produce phenotypic expressions of heterokaryosis, but insufficient to be contributory to actual conidium formation, hence undemonstrable by the techniques currently employed.

The medium upon which mutant strains are intermixed obviously exerts a profound effect upon their behavior relative to one another. This fact is readily demonstrated. For example, large black heterokaryotic heads regularly develop in the dense and strongly intermixed central area of colonies on high-sugar and Czapek agars in pairings of *Cinnamon* or *Clay* × *Brown*, whereas they develop sparingly or not at all along colony interfaces on steep agar where the two mutants form well-defined, rapidly spreading, plane wedges of growth. The enhancement of heterokaryon formation in the former media is presumed to result from more richly branched mycelia resulting in greatly increased hyphal anastomoses with concomitant nuclear interchange.

The patterns of heterokaryons produced in *Clay* × *Fuzzy*, the yellow *Cladosarum*-like mutant from *Orange*, were essentially like those earlier described in mixtures of *Brown* × *Fuzzy*, and ranged from large, globose heads with long chains of jet-black conidia to smaller structures wherein the nonsporulating cellular elements of *Fuzzy* were seen to project beyond the mass of well-formed black conidia. Upon cultural analyses, heads of the latter type generally yielded somewhat more strongly heterokaryotic populations than the former.

Intermixtures of Mutants from Different Strains

The preceding studies involved the intermixture of mutants derived directly or indirectly from a single strain of *A. fonsecaeus*, NRRL 67. It seemed of interest, therefore, to produce and investigate to a limited degree the interaction of mutants derived from other original sources. Basic stocks employed for this phase of the investigation are listed on page 6 and include the following: NRRL 591, NRRL A-2589, and NRRL A-1310. All should probably be diagnosed as representing *A. fonsecaeus*, as this species is currently understood; certainly they would represent members of the large-spored *A. carbonarius* series of the *A. niger* group as presented by Thom and Raper (1945). Of these three cultures, A-2589 is especially interesting since it represents a nitrate deficient strain isolated directly from nature.

Early in this study, the stock culture of NRRL 591, a second large-spored black *Aspergillus* obtained from Brazil, was planted in apposition to *Tan*, the first of the ultraviolet-induced and lightly pigmented mutants of NRRL 67. Heterokaryotic heads of intermediate coloration (blends) were formed at colony interfaces. Conidial analyses of such heads revealed a situation similar to that already observed in mixtures of *Tan* × NRRL 67 (*Stock*). This observation is important to the present study in that it provided the first evidence that heterokaryons could be established between strains of completely different origins.

Mutations of NRRL A-2589 and A-1310 were produced by ultraviolet radia-

tion and isolated by the same methods as already considered, and pairings were made by implanting conidia of selected mutants in the conventional way on steep agar. Other media or methods of inoculation were not employed, a circumstance which may have limited the number of cases in which heterokaryon formation could be demonstrated (see p. 18). In all cases where true heterokaryons were

MUTANTS	A-2589-28-2	A-2589-28-3	A-2589-55-1	A-2589-55-3	A-2589-50-1	A-1310-28-1	A-1310-28-2	A-1310-28-4	A-1310-28-5	A-1310-28-6	A-1310-28-11	A-1310-55-6	A-1310-55-8	Orange	Brown	Fuzzy
A-2589-28-2: Brown mutant with small conidial heads																
A-2589-28-3: Brown mutant with large conidial heads	●															
A-2589-55-1: Almost sterile, white mycelium; few small black heads	●	○														
A-2589-55-3: Orange-tan mutant	●	○	◐													
A-2589-50-1: Predom. sterile, yellowish mycelium; few small heads	●	●	●	●												
A-1310-28-1: Brown mutant, conidial heads fairly large	○	○	○	○	○											
A-1310-28-2: Cladosporium-like, dark gray	○	○	○	○	○	●										
A-1310-28-4: Tan mutant	○	○	○	○	○	○	●									
A-1310-28-5: Tan mutant	○	○	○	○	○	○	●	○								
A-1310-28-6: Cladosporium-like; light gray-green	○	○	○	○	○	○	○	○	○							
A-1310-28-11: Orange mutant	○	○	○	○	○	●	●	●	●	●						
A-1310-55-6: Tan mutant, recombines A-1310-45-8	○	○	○	○	○	○	●	○	○	○	●					
A-1310-55-8: Tan mutant, recombines A-1310-45-6	○	○	○	○	○	○	●	○	○	○	●	○				
Orange	○	○	●	●	●	○	○	○	○	○	○	○	○			
Brown	○	○	●	●	●	○	○	○	○	○	○	○	○	●		
Fuzzy	○	○	●	●	●	○	○	○	○	○	○	○	○	○	●	

● Black heterokaryons: heads and conidia.

◐ Blends: heterokaryotic heads and conidia.

○ Heterokaryons not observed.

DIAG. 5. Patterns of behavior observed when selected mutations from three different original cultures of *A. fonsecaeus* are paired in all possible combinations. Note especially the capacity of *Brown*, *Orange* and *Fuzzy* (derived from NRRL 67) to heterokaryotise with certain mutations of NRRL A-2589. (See discussion in text.)

produced these appeared as black or near black conidial heads, invariably situated at or near the interfaces of the paired mutant colonies. Occasional structures which suggested blends failed to reveal heterokaryosis, representing instead one or the other of the mutant types which had apparently been vegetatively stimulated by the presence of the second mutant. Conidial analyses of presumptive heterokaryotic heads were made generally by dilution platings on steep and Czapek agars. Results are summarized in Diag. 5.

Certain significant facts are revealed. It is further demonstrated that hetero-

karyons can be produced by pairing cultures (or their progeny) which stem from different original sources. This is clearly shown in the behavior of *Brown*, *Orange*, and *Fuzzy*, mutants derived from NRRL 67, planted in apposition to three mutants of NRRL A-2589. It would seem equally clear that, using present techniques, such heterokaryons of different basic origins are the exception rather than the rule. Not only did *Brown*, *Orange*, and *Fuzzy* fail to intermix with most of the mutants investigated, but, up to this time, we have had no success in intermixing any mutants derived from NRRL A-2589 with those from NRRL A-1310.

Upon further analysis, it is equally apparent that certain mutants obviously possess a much greater capacity to form heterokaryons than others, a potentiality already revealed by *Orange* and its derivatives among the mutants of NRRL 67 (Diag. 3). In the present tests, for example, among the five mutants derived from NRRL A-2589, strains *2589-28-2* and *2589-60-1* formed heterokaryons with all others, whereas of the remaining three only *2589-45-1* and *2589-45-3* formed heterokaryons when paired among themselves. These same mutants, together with *2589-60-1* formed heterokaryons with three selected mutants derived from NRRL 67, whereas *2589-28-2*, which intermixed actively with its sister mutants, failed to do so (Diag. 5). Crosses between strains *2589-45-1* and *2589-60-1* were particularly striking since both mutants represent nearly sterile forms, producing only scattered and very small conidial structures. Intermixed colonies, on the other hand, produce abundant large, black conidial structures on substrata such as steep agar. Recultivation from such colonies has yielded strongly heterokaryotic cultures which can be maintained in this state with singular ease since essentially all of the conidia are produced in heterokaryotic heads and an unusually high proportion of individual conidia carry nuclei of both mutant forms.

Much the same overall picture is presented by the mutants of NRRL A-1310. Two of these, namely, *1310-28-2* and *1310-28-11*, formed heterokaryons with six and all of seven sister mutants, respectively, whereas the remaining six mutants failed to form any heterokaryons when paired among themselves (Diag. 5). In contrast with three of the mutants obtained from NRRL A-2589, none of those derived from NRRL A-1310 formed heterokaryons with the three test mutants from NRRL 67. Failure of *1310-28-2* to form heterokaryons with *1310-28-6* is interesting, but not surprising, since both strains represent mutations of the *Cladosarum* type. These forms, characterized by marked structural abnormalities, are presumed to possess more deep-seated differences than mutants which differ primarily in pigmentation or nutritional deficiencies. In mixtures with *1310-28-2*, heterokaryotic heads are comparatively large and black. Attempts to intermix this with *Fuzzy*, the bright yellow *Cladosarum*-like mutant from NRRL 67, have to date been unsuccessful.

INTERMIXTURES IN OTHER SPECIES

While we have not made a detailed study of the phenomenon of strain intermixture in any species consistently producing uninucleate conidia, we have

undertaken a limited cultural and cytological examination of *A. clavatus* Desm. Strains employed in this work represent a normal blue-green strain (NRRL 2254) which is wholly typical of the species, and a white-spored mutant (of natural origin) which apparently differs from the parent only in its complete lack of conidial color (NRRL 2255). When these two forms are planted together, mixed colonies are produced in which develop abundant blue-green heads typical of the parent, an approximately equal number of pure white heads, and scattered intermediate, or heterokaryotic, heads. The latter duplicate the other types in size and pattern but differ in color, ranging from pale blue-green, through light gray to off-white. Upon cultural analysis individual conidia are found to develop either pure white or pure blue-green colonies, but never mixtures of the two.

Stained preparations of *A. clavatus* made by the techniques previously cited show the sterigmata, which occur in a single series only, to be consistently uninucleate, as are also the conidia arising from them. (See Yuill, 1950.) While we have not separated the conidia of individual chains and subsequently checked the colonies developing from them, we feel confident that individual sterigmata give rise to chains of like conidia, either blue-green or white. Viewed with the low-power binocular microscope the heads sometimes appear to be lightly striate. Viewed under high magnifications, the coloring of the conidia in the blue-green parent is so slight as to be hardly evident, and is quite insufficient for distinguishing the parent from the mutant. The heterogeneity of conidial heads produced in *A. clavatus* would appear to be strictly comparable with that reported by Gossop and the Yuills (1940) for some of the cultures of *A. niger* investigated by them.

Studying mixed colonies of a normal, cinnamon-colored strain of *A. terreus* Thom and a colorless mutant (of natural origin) isolated from it, we have observed striped heterokaryotic conidial heads which clearly are composed of some chains of white and other chains of colored conidia. Individual spores give rise to pure colonies either of the pigmented parent or the albino mutant. The same pattern of behavior has been observed in mixed plantings of a normal green-spored strain of *A. fumigatus* Fres. and a naturally occurring albino mutant obtained from it. Heterokaryotic heads are conspicuously striate, being composed of white and green conidial chains. Individual conidia yield colonies of the parent or the mutant. Behavior in these species follows the pattern of development described and illustrated by Gossop *et al.* (1940) for *A. nidulans*, and would represent excellent examples of "autonomous" nuclear action in heterokaryons according to Pontecorvo's interpretation (1946).

DISCUSSION

Three distinct types of demonstrable heterokaryotic heads have been encountered when different mutants, or mutant and stock cultures of selected strains of *A. fonsecaeus* are grown in mixed or contiguous culture. Heterokaryotic heads of intermediate pigmentation (blends) were regularly produced in intermixtures of the black parent (*Stock*) or a black-spored nitrate deficient mutant (*Thin*) with some mutant of lighter color (*e.g. Tan* or *Orange*, of Diag. 3). Such

intermediate conidial pigmentation is believed to represent simply a dilution of black by the less highly colored mutant. Black heterokaryotic heads, generally indistinguishable from the wild type, were produced in intermixtures of two lightly colored mutants (*e.g.*, *Orange* × *Brown* or *Tan*, etc. of Diag. 3). Reappearance of black in such cases is interpreted to result from the reconstitution within a single fruiting structure of two factors (or sets of factors) essential for the production of black pigment, one of these being absent from each of the two paired mutants. Gray-brown heterokaryotic heads were produced in a limited number of cases when primary and secondary, or two secondary mutants, were planted in apposition to each other (*e.g.*, *Cinnamon* × *Tan* or *Pink*, of Diag. 4). Elaboration of this gray-brown pigmentation in heterokaryotic heads could result from two mutants providing partial but incomplete factors necessary for the production of black pigment. Interestingly enough, all examples which show this behavior involve color (secondary) mutations of *Thin* growing in mixed culture with or contiguous to *Tan*, or secondary mutations derived from it.

No satisfactory explanation has been found for the large black (and presumptively heterokaryotic) heads which, upon dilution plate analyses, yield only pure colonies of one mutant, usually *Brown* (*e.g.*, *Tan* × *Brown*, *Cinnamon* × *Brown*, etc.). They can be interpreted as resulting from mycelia and developing fructifications which contain such limited numbers of nuclei of the opposed mutant that these never reach the sites of conidium formation. But proof of this is not at hand. We do know that ratios of nuclear types can vary greatly in the conidia of demonstrably heterokaryotic heads, and in mixed heads from such combinations as *Brown* × *Cinnamon* ratios as disproportionate as 25:1:1 for pure *Brown*, heterokaryotic, and pure *Cinnamon* colonies, respectively, have been obtained from some conidial heads by dilution plating, and by single spore analyses. Can such imbalance of nuclear types be projected substantially further? If, for example, pure *Brown* occurred in a ratio of 100^n:1:1, it is quite possible that the presence of an occasional heterokaryotic spore would escape detection.

Mutant strains differ markedly in their inherent capacity to produce heterokaryons, as seen in Diags. 3 and 5, and the quantity of such structures ranges in different combinations from very abundant, forming nearly continuous lines along colony interfaces in some cases, to occasional isolated heads in others, to a complete absence in still others.

Additionally, the manner in which two mutants are intermixed and the substratum upon which they are implanted markedly influences the production of heterokaryotic fruiting structures. For most of our work, paired strains have been inoculated on steep agar as described earlier with a view to securing well-defined frontiers between mutant colonies to facilitate the indentification of heterokaryotic heads which might develop. The use of mixed spore suspensions as spot inoculations on Czapek and high-sugar agars has in some instances yielded heterokaryotic heads when none were observed in plates of the preceding type (*e.g. Cinnamon* × *Brown*). In the conjoint behavior of two strains, factors such as relative growth rates and nutritional deficiencies, which characterize many of them, play an important role. If each combination of strains could have been

intermixed under a wider variety of conditions, heterokaryons would probably have been obtained in some instances now recorded as negative. Nevertheless, we believe our techniques have provided a reasonably accurate estimate of their inherent capacity to heterokaryotise. Irrespective of the conditions under which they were first obtained, we have encountered no difficulty whatever in maintaining any heterokaryon once it was established.

Marked stimulation of one mutant by a contiguous one is rather commonplace and apparently quite independent of any phenomenon of heterokaryosis. Such stimulation is generally evinced in a narrow band of conidial heads of one mutant at a mutant-mutant interface, these structures being two or three times the diameter of other heads located well within the quadrant of the pure strain. No known correlation with any nutritional deficiency has been demonstrated, nevertheless we regard the response as basically nutritional and to result from the more ready availability of some factor produced by one mutant and stimulatory to the other.

The nuclear condition and pattern of developmental behavior in mixtures of mutant, or parent and mutant, strains of *A. fonsecaeus* is strikingly similar to those reported by Hansen and co-workers for various representatives of the Fungi Imperfecti and referred to as the "dual phenomenon" (Hansen, 1938; Hansen and Snyder, 1943). Unlike certain of the species investigated by them, no known element of sexuality enters into the mixtures of *A. fonsecaeus*. As a matter of fact, the production of perithecia is unknown for the *A. niger* group and no mechanism was perceived whereby nuclear fusion might be expected to occur. Nevertheless, we endeavored to be alert to any developments which might suggest such a possibility. This was especially true following publication of the highly significant work of Roper (1952) wherein he obtained from heterozygous diploids in *A. nidulans* (an ascosporic species) "rare somatic recombinations" resulting in diploids homozygous for one or more markers. Aside from the repeated observation and analysis of black heads which yielded only pure colonies of one of two opposed mutants (usually *Brown*), we have encountered no behavior which cannot be satisfactorily explained in terms of simple nuclear migration and intermixture. And in that case, our present lack of understanding may result from the inadequacy of our experimental techniques.

The capacity of mutant strains of *A. fonsecaeus* to form heterokaryons may point to potentially important practical applications. Strain NRRL 67 has long been recognized as a good producer of citric (Wells *et al.*, 1936) and gluconic (Moyer *et al.*, 1937) acids. If mutants possessing improved and different characteristics pertaining to one, or both, of these fermentations could be obtained, and if these could be induced to heterokaryotise, could we not reasonably expect to reap the benefits of heterokaryotic vigor (Dodge, 1942; Beadle and Coonradt, 1944)? The possession by such mutants of altered pigmentation could vouchsafe the perpetuation of a heterokaryotic culture with minimal effort. Application of this principle to most fermentative molds would be more difficult since most of them produce uninucleate conidia. The potential, however, still exists—it being necessary in such a case to work out conditions for periodic reproducible re-

constitution of the heterokaryotic culture. Such a scheme was proposed several years ago by Lindegren and Andrews (1945) for the penicillin fermentation. Their experiments failed of immediate practical significance—perhaps because they were not working with strains possessing optimal biosynthetic characteristics, and perhaps equally because they introduced no markers (*e.g.* pigmentation) by which the degree of heterokaryosis could be gauged. More recently, Yuill (1951) has reported some preliminary investigations of citric acid fermentations with mixed strains of *A. niger* without overall improvement in acid production. The extent to which the mixed cultures heterokaryotised was not discolosed. Despite these two essentially negative reports, the authors believe this to represent a potentially rewarding approach.

SUMMARY

When mutant strains, or mutant and parent strains, of *Aspergillus fonsecaeus* Thom and Raper are grown in juxtaposition, heterokaryotic conidial structures commonly develop along the interface between the two colony areas. Such heterogeneous fructifications arise as a result of hyphal anastomoses and nuclear intermixture between the adjacent mycelia. Conidiiferous cells are multinucleate, and the heterokaryotic condition commonly extends into the conidia, or reproductive elements. Characteristically, these give rise to three colony types, representing pure colonies of each mutant (or mutant and parent) and mixed colonies containing heterokaryotic heads and conidial structures typical of each mutant.

In mixtures of a lightly pigmented mutant and the black parent (or of such a mutant and one with normal black pigmentation), heterokaryotic heads appear in shades of pigmentation intermediate between the opposed strains. In mixtures of two lightly pigmented strains, heterokaryotic heads are usually, but not always black, simulating the wild type. In some combinations of lightly pigmented mutants, large black heads (presumptive heterokaryons) develop, which, upon conidial analysis, yield only pure populations of a single mutant. This phenomenon is not completely understood and some possible explanations are considered.

Mutant strains vary greatly in their capacities to form heterokaryons, some combinations producing these abundantly, some sparingly, and others not at all. Conditions of cultivation markedly influence the degree of heterokaryosis achieved. However, once a mixed head is produced, no difficulty is experienced in maintaining the heterokaryotic condition through subsequent recultivations.

The formation of heterokaryotic heads in mixed plantings of parent and albino strains has been demonstrated in *A. clavatus* Desm., *A. fumigatus* Fres. and *A. terrus* Thom. These species produce uninucleate conidia, and the pattern of development coincides with that previously reported by English investigators for other species.

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ON CERTAIN WELL-ORDERED MONOTONE COLLECTIONS OF SETS*

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This paper is concerned with a generalization of the following theorem in the theory of sets:

Suppose that for each positive integer n, H_n is a finite collection of point sets such that each point set of H_{n+1} is a subset of some point set of H_n. Then there exists a sequence $h_1, h_2, h_3, \cdots$ such that for each n, h_n belongs to H_n and h_{n+1} is a subset of h_n.[1]

The "point sets" mentioned in the theorem are presumed to be subsets of a certain space S, but since no use is made of the topology of S, the theorem is purely a theorem of logic which may be stated in other terminology (*e.g.*, the terminology of partially ordered sets or lattice theory).[2] If H_n were allowed to be *infinite*, the conclusion would not follow. This would be the case if for each positive integer n, H_n were the collection of open real number unit-intervals $(n, n+1); (n+1, n+2); (n+2, n+3); \cdots$. However, the theorem may be generalized as indicated below. Certain inessential complications may be avoided by assuming that the sets in H_n are mutually exclusive, and this will be done for the sake of simplicity.

Theorem. *Suppose that z is an ordinal number and F is a well-ordered family of collections $H_1, H_2, H_3, \cdots, H_x, \cdots, x < z$, of mutually exclusive point sets such that* (1) *if F' is a subsequence of F running through F, $|F'| = |F|$,* (2) *if F' is a subsequence of F which does not run through F, $|F'| < |F|$,* (3) *if $x < y < z$, each point set of H_y is a proper subset*[3] *of some point set of H_x, and* (4) *there exists a cardinal number N such that for each $x < z$, $|H_x| < N < |F|$. Then there exists a sequence $h_1, h_2, h_3, \cdots, h_x, \cdots$ such that for each x $(x < z)$, h_x belongs to H_x, and if $x < y < z$, h_y is a subset of h_x.*

Proof.[4] Let F' denote the sequence: $, H_1', H_2', H_3', \cdots, H_x', \cdots (x < z)$, such that for each x, H_x' consists of those elements of H_x which contain $|F|$ elements of F^*.

Each element of $(F')^*$ contains $|F|$ elements of $(F')^*$. For suppose on the contrary that some element h of $(F')^*$ contained *less* than $|F|$ elements of $(F')^*$. Let x be the smallest ordinal such that H_x contains such an element h, and let Q

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[1] *Cf.* R. L. Moore, *Foundations of Point Set Theory*, American Mathematical Society Colloquium Publications **13:** New York, 1932, Theorem 67, p. 54. Also see Ramsey [8] and König [2].

[2] In particular see [1].

[3] The theorem is true if the word *proper* is omitted. The same argument as given here applies with changes in phraseology only.

[4] In this paper if F denotes a collections of sets, F^* denotes the sum of the elements of F.

denote the elements of F^* contained in h. Then $|Q| = |F|$. Let Q_{x+1} denote the elements of H_{x+1} contained in h. Since $|Q_{x+1}| < |F|$, $|Q - Q_{x+1}| = |F|$ and each element of Q is a subset of some element of Q_{x+1}, it follows that some element of Q_{x+1} contains $|F|$ elements of Q. Hence some element of Q_{x+1} belongs to $(F')^*$. A similar argument shows that if Q_{x+y} $(y < z)$ is the set of elements of H_{x+y} contained in h, then Q_{x+y} contains an element of $(F')^*$. Hence h contains $|F|$ elements of $(F')^*$.

Consequently, in what follows it is assumed that F possesses the property just shown to be valid for F', namely: each element of F contains $|F|$ elements of F^*.

Now suppose that the theorem is false. Let h_1 denote an element of H_1. If for each x $(1 < x < z)$, H_x has only one element in h_1, then h_1 together with this one element from each H_x, for each x $(1 < x < z)$, would form the sequence required in the theorem. This being contrary to assumption, there exists an ordinal x_1 such that H_{x_1} has *two* elements h_1^1 and h_1^2 which are subsets of h_1. Let h_2 be an element of one of the sets H_x following H_1 and H_{x_1} in F. Again there exists an ordinal x_2 such that two elements h_2^1 and h_2^2 of H_{x_2} each of which lies in h_2. Continue this process N times so as to obtain a sequence α of elements h_1, h_1^1, h_1^2, h_2, h_2^1, h_2^2, $\cdots$ such that (a) if h_x belongs to α, it belongs to an element H of F which follows in F every element of F which contains an element of α preceding h_x in α, and (b) h_x contains two sets h_x^1 and h_x^2 which belong to an element of F which follows H in F. Now let H_y denote the first element in F such that H_y follows in F every element of F which contains an element of α. Since $|\alpha| = N < |F|$, such an element must exist. For each element h of H_y let β_h denote the set of all elements of F^* which contain h. *If* β_h contains an element h_x of α it cannot contain both h_x^1 and h_x^2. So if β_h contains more than $|H_y|$ elements of α it fails to contain more than $|H_y|$ mutually exclusive elements of α and each of these must contain an element of H_y. This is impossible. Hence, for each h of H_y, β_h contains at most $|H_y|$ elements of α. Since $\sum \beta_h$ $(h \in H_y)$ contains every element of α, it follows that $|\alpha| \leqq |H_y|^2$. But[5] $|H_y|^2 < N$. So $|\alpha| < N$, which is a contradiction.

If the inequality in (4) of the theorem is relaxed to read $|H_x| \leqq N < |F|$, the resulting proposition is false. The simplest case of this is illustrated in the following example.

CONSTRUCTION.[6] Let G be the collection of all well-ordered sequences g of positive integers such that (a) g has a last term, (b) g is of ordinal type less than ω_1, and (c) no integer appears in g infinitely many times. For each g of G, let $h(g)$ denote the set of all elements g' of G such that g is an initial segment of g', and let H denote the collection of all such sets $h(g)$ $(g \in G)$.

Now let H_1 denote the subcollection of H consisting of all of those elements

[5] Since the theorem is assumed to be false, N may be considered to be transfinite, for otherwise the proposition is a corollary of the theorem of the opening paragraph.

[6] The following construction and example were presented to the Mathematics Club of the University of Texas in an address entitled, *A false proposition of logic*, in the Spring of 1933.

$h(g)$ of H such that g belongs to G and has only one term. Let H_2 denote the subcollection of H consisting of all of those elements $h(g)$ of H such that g belongs to G and has exactly two terms. Suppose this process to be continued such that (1) for some ordinal $z < \omega_1$, H_x has been defined for each ordinal $x < z$, (2) for each ordinal $x < z$, H_x is a countable subcollection of H such that if $h(g)$ $(g \in G)$ belongs to H_x, then g is of ordinal type $1, 2, 3, \cdots, x$, (3) if $x < y < z$, then each element of H_y is a subset of exactly one element of H_x, and (4) if $x < y < z$, n is a positive integer and $h(g)$, $g \in G$, is an element of H_x, then there exists an element g' of G such that (i) $h(g')$ belongs to H_y, (ii) $h(g')$ is a subset of $h(g)$, and (iii) every term of $g'-g$ is greater than n.[7] The collection H_z is now defined in one of two ways: I. If z has an immediate predecessor, then H_z consists of all elements $h(g)$ of H such that $h(g)$ is a subset of some element of H_{z-1} and g is of ordinal type $1, 2, 3, \cdots, z$; II. If z has no immediate predecessor, select a countable monotone increasing sequence $z_1, z_2, \cdots, z_i, \cdots$ of ordinals whose least upper bound is z. Suppose that n is a natural number and that g_1 is an element of G such that $h(g_1)$ belongs to H_{z_1}. It follows from (4) that there exists g_2 of G such that $h(g_2)$ belongs to H_{z_2}, $h(g_2)$ is a subset of $h(g_1)$ and every term of $g_2 - g_1$ is greater than $n + 1$. Similarly, there exists g_3 of G such that $h(g_3)$ belongs to H_{z_3}, $h(g_3)$ is a subset of $h(g_2)$ and every term of $g_3 - g_2$ is greater than $n + 2$. If this process is continued, the sequence of integers determined by the sequence $g_1, g_2 - g_1, g_3 - g_2, \cdots, (n + 1)$ belongs to G and is of ordinal type $1, 2, 3, \cdots, z$. Since the collection G' of all elements g' of G such that $h(g')$ belongs to one of the collections $H_{z_1}, H_{z_2}, \cdots, H_{z_i}, \cdots$ is countable, each such g' of G' may be assigned an infinite sequence $\alpha(g')$ of positive integers such that no integer belongs to two such sequences $\alpha(g')$. For each element g' of G' and each integer n of $\alpha(g')$ select one element g of G (whose existence is shown above) of ordinal type $1, 2, 3, \cdots, z$ and let the collection of H_z consist of all sets $h(g)$ for each such g. This completes the definition of $H_1, H_2, \cdots, H_z, \cdots (z < \omega_1)$ by induction since H_z as defined by either I or II has properties (1) – (4) above.

Furthermore, suppose there exists a sequence $g_1, g_2, \cdots, g_z, \cdots (z < \omega_1)$, such that $h(g_1) \supset h(g_2) \supset h(g_3), \cdots$ and for each z, $h(g_z)$ belongs to H_z. It follows that some positive integer n must appear infinitely many times in the sequence of integers determined by $g_1, g_2 - g_1, \cdots, g_{z+1} - g_z, \cdots$. Hence for some z, n must appear in the sequence g_z infinitely many times. Consequently, this being contrary to the definition of G, g_z is not an element of G. So no such uncountably infinite sequence $g_1, g_2, \cdots, g_z, \cdots (z < \omega_1)$ exists. From this argument and the preceding construction, the following example results.

EXAMPLE 1. *There exists a well-ordered family F of collections $H_1, H_2, \cdots, H_z, \cdots (z < \omega_1)$ such that (1) for each ordinal x, $x < \omega_1$, H_x is a countable collection of mutually exclusive sets, (2) if $x < y < \omega_1$, each set of H_y is a proper subset of a set of H_x, (3) if $x < y < \omega_1$, and h_x is an element of H_x, there exists a sequence $h_x, h_{x+1}, h_{x+2}, \cdots, h_y$ such that $h_x \supset h_{x+1} \supset h_{x+2} \supset \cdots \supset h_y$, and for each z, $x \leqq z \leqq y$, H_z contains exactly one of the sets $h_x, h_{x+1}, h_{x+2}, \cdots, h_y$,*

[7] Since g is an initial segment of g', the meaning of $g'-g$ is evident.

but (4) *there exists no sequence* $h_1, h_2, h_3, \cdots$ *such that* $h_1 \supset h_2 \supset h_3 \supset \cdots$ *and for each* x, $x < \omega_1$, H_x *contains exactly one of the sets* $h_1, h_2, h_3, \cdots$.

Application. Suppose that F is a sequence described in Example 1. If for some x, $x < \omega_1$, h is a set of H_x, let h be called a "point." If h belongs to an H_x such that $x - 1$ exists, let h be a "region." If h belongs to H_x such that $x - 1$ does *not* exist, then for each $y < x$, let the sequence $h_y, h_{y+1}, h_{y+2}, \cdots, h$ such that $h_y \supset h_{y+1} \supset h_{y+2} \supset \cdots \supset h$, and for each z, $y \leqq z \leqq x$, H_z contains exactly one of the sets $h_y, h_{y+1}, h_{y+2}, \cdots, h$, be a "region." Let S denote the collection of "points" thus obtained. The "space" S satisfies R. L. Moore's Axioms 0 and 1.[8] Hence S is a complete Moore space. Furthermore, S is the sum of the elements of a sequence $D_1, D_2, \cdots, D_x, \cdots$ $(x < \omega_1)$ of perfectly separable domains (in fact, in this case, if one lets D_x consist of all elements of H_x together with all elements of each term of F which precedes H_x, then D_x contains only countably many "points") such that each $\bar{D}_x$ is a proper subset of each D_z which follows it. It follows from one of Alexandroff's theorems that S is *not metric.*

As a matter of fact, by replacing the "points" with straight line segments (open intervals), by joining each pair of these intervals which correspond to a pair h_1, h_2 of "points" with rectangular plane domains when the set $h_1 \supset h_2$ and they belong to consecutive H_x's, and by extending the notion of region, a space is obtained which has the properties just mentioned for S but which satisfies Moore's Axioms 0, 1, 2, 3, 4, 5_1, 5_2, and 6. Consequently, this space is very much like an Euclidean plane. It is locally a plane, cyclically connected, satisfies the Jordan curve theorem, and all but at most one of the complementary domains of a compact continuum are themselves compact. Nevertheless, the space contains an uncountable increasing sequence of perfectly separable connected domains, each lying together with its boundary in all succeeding domains.

Application to partially ordered sets. If P denotes the partially ordered set whose elements are the *sets* of Example 1, and $x < y$ if and only if x contains y, then P has the following properties:

(1) $|P| = \aleph_1$,

(2) if $x \in P$ and $S(x)$ is the set of all elements y of P such that $y \leqq x$, then $S(x)$ is well-ordered and $|S(x)| = \aleph_0$,

(3) if $x \in P$, there are uncountably many elements y of P such that $x < y$,

(4) if $x \in P$, the set of all elements y of P such that $S(y)$ is of the same ordinal type as $S(x)$ is countable,

but (5) every ordered subset of P is well-ordered and countable.

Remarks. The sequence $F = H_1, H_2, \cdots$ of Example 1 has the property that $|F| = \aleph_1$. If F is such a sequence but $|F| > \aleph_1$, an example similar to Example 1 may be constructed for F provided that some subsequence F' of F runs through F and $|F'| = \aleph_1$. Each collection in F–F' could be defined to be identical with the first collection of F' following it in F. This observation in not new [2].

This proposition gives, in many cases, Helson's [4] negative answer to Sikor-

[8] The sequence $G_1 G_2, G_3, \ldots$ of collections of regions may be defined in a rather natural way with the help of the numbers in the elements of G in the construction preceding Example 1.

ski's problem[9] even under the restrictions imposed by Specker [9]. It should be pointed out that the case settled by Specker (corresponding to $| F | = \aleph_1$) could have been settled by using an example due to Aronszajn reported by Kurepa [6, p. 96; 7].

Aronszajn's example is similar to the one I have constructed. It yields Example 1 but is not as suitable for the application to Moore spaces. My construction was discovered while trying to settle the question: Is every normal Moore space metric? [5] This question still remains unanswered. In fact, it is not known whether a non-metric Moore space of the type described in the application is normal or not.

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THE CONVERSION OF CYSTEIC ACID TO TAURINE

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The relations between taurine and cysteic acid have been of interest since Friedmann (1903) pointed out the similarity of their structure. Moreover the ease with which the latter can be formed from cystine or cysteine by even mild oxidizing conditions (Andrews, 1932, 1933) have made cysteic acid appear as a very plausible intermediate. Friedmann reported a 59% *in vitro* conversion of cysteic acid to taurine by heating the former under pressure at about 235°C. for two hours. Later attempts to repeat his work have met with little success (Gortner and Hoffman, 1927; Lewis and Lewis, 1926). Only White and Fishman (1936) reported yields as high as 72% of taurine from cysteic acid. They attributed their success to very close temperature control (between 235° and 240°C.), implying either that taurine must suffer further decomposition above 240° or that other reactions occur if higher temperatures are quickly reached.

The metabolism of cysteic acid has been variously reported. There is some evidence that it can be a precursor of taurine (Virtue and Doster-Virtue, 1939) and ample evidence that it is incapable of producing urinary sulfate. Cysteic acid decarboxylase has been reported by Blaschko (1942), Medes and Floyd (1942) and Blaschko *et al.* (1948) but its characterization is not as clear as could be desired.

Methods

Only recently has the widespread occurrence of taurine in small amounts been realized, largely as a result of applying the technique of paper chromatography for its detection (Dent, 1947; Roberts and Frankel, 1949). As yet, however, there exists no simple and satisfactory colorimetric method for the determination of taurine in biological materials comparable to the methods which have been developed for amino acids.

Colorimetric Method for Taurine—The quantitative method of Schick and Degering (1947) involves serious interference by other amino derivatives. However, we have adapted this method to low concentrations by carefully controlling the proportion of reagents, especially the oxidant, as described below:

One to four ml. of the unknown solution, containing from 0.003 to 0.02 millimoles of taurine, were measured into a large test tube together with 3.0 ml. of 1% phenol. A series of samples of standard 0.01 M taurine was also measured in such amounts as to bracket the expected amount in the unknown solution. A blank was also prepared, using a volume of distilled water equal to the largest standard sample. After the addition of 0.5 ml. of 5.2% sodium hypochlorite (Chlorox) to each tube in rapid succession (producing the desired pH 9.4), all tubes were allowed to stand at room temperature for exactly 10 minutes. During

this time a noticeable color developed in all tubes except the blank. At the end of the 10 minute period all tubes were placed in a rack and immersed simultaneously in a boiling water bath for exactly 3.5 minutes. Upon removal they were chilled at once in cold running water and made up to 10 ml., beginning with the one to which hypochlorite was first added and diluting all in the original order. As soon as these tubes regained room temperature, samples were removed and read in the spectrophotometer at 625 mμ. By this procedure a minimum of 3.0 micromoles taurine at a concentration of 0.5 millimoles per liter may be determined with an accuracy of $\pm$ 5 to 10%. At lower concentrations erratic results are produced by traces of ammonia in the air. Average recoveries on known solutions of taurine from the amounts mentioned above up to over 100 micromoles approached 100 $\pm$ 4%. The reaction is useful as an analytical tool for taurine only when no other ammonia derivatives are present.

Chromatographic Methods—The possibilities of chromatographic separation and estimation of amino acids and related compounds have been explored by a number of investigators such as Dent (1947), Consden, Gordon and Martin (1944), Awapara (1948, 1949), Landua and Awapara (1949), and Martin and Mittelmann (1948) with the conclusion that the ninhydrin reaction is capable of yielding acceptable results from chromatographic separations if conditions are closely controlled. We have therefore applied a combination of the techniques of other workers.

The first chromatograms were qualitative and were run according to the ascending method of Williams and Kirby (1948) using as the solvent 80% phenol and 20% water (Bull, Hahn and Baptist, 1949) in one-dimensional chromatograms, and in two-dimensional chromatograms 2,4,6-collidine saturated with water. The chromatogram, after being sprayed with ninhydrin, was dried at a temperature not over 40°C. to develop the maximum color and was then interpreted by comparison on the same chromatogram of the unknown solutions for which the R_f numbers had been determined.

In the quantitative work a sample of unknown was run on the same sheet with a standard containing known amounts of each component at concentrations approximating those in the unknown sample. The spots were eluted with hot distilled water and the solution used in a colorimetric determination. This procedure was used for the undecomposed cysteic acid, the colored solution resulting from ninhydrin treatment being read in a spectrophotometer at 570 mμ. For taurine it was found that the ninhydrin reaction was inferior to the phenol-hypochlorite reaction.

Other substances resulting from cysteic acid decarboxylation were determined by conventional methods. These included carbon dioxide, ammonia, sulfuric acid and sulfur. Lactic acid was determined by the method of Barker and Summerson (1941). Alanine was determined directly by the ninhydrin reaction when there was no other interference. When small amounts of lactic acid and alanine were both present the method described by Block and Bolling (1951) for the production of acetaldehyde from the two was followed by the Barker and Summerson procedure.

After a series of exploratory experiments designed to reveal optimum conditions for a qualitative study, a series of runs was made in a Carius tube furnace. 2.0 g. cysteic acid in 20 ml. water were sealed in a Carius tube with about 30 ml. of free space for expansion. After heating, the tubes and contents were frozen by solid carbon dioxide before opening. After opening, the gas from the tube was passed with as little loss as possible into barium hydroxide solution and carbon dioxide determined in the usual way. In the runs at higher temperatures, where there was more reduction of sulfur, there were indications that some carbon dioxide was produced from carbon atoms other than the carboxyl, since the molar yield of carbon dioxide was sometimes decidely higher than that of taurine.

Table I shows the results of a number of Carius tube runs. Column 3 shows the average temperature attained during the heating. Columns 4 to 8 inclusive give the amounts of undecomposed cysteic acid and of taurine, alanine, ammonia and

TABLE I

Results of heating water solutions of cysteic acid in Carius tubes in proportion of 2.0 gm. (11.8 millimoles) per 20 ml. water

RUN NO. (1)	TIME (2)	TEMP. (3)	CYSTEIC ACID UNCHANGED (4)	TAURINE (5)	ALANINE (6)	AMMONIA (7)	SULFURIC ACID (8)	LACTIC ACID (9)	CARBON DIOXIDE (10)	TOTAL SOLIDS DET'N'D (11)	TOTAL SOLIDS WEIGHED (12)	PER CENT RECOVERY OF TOTAL SOLIDS (13)
	Hrs.	°C.	*mM.*	*mM.*	*mM.*	*mM.*	*mM.*	*μM*	*mM.*	*mg.*	*mg.*	%
1	5.0	190	9.35	0	0.12	0.04	0.03	0.70	0	1594	1800	88
2	5.5	200	10.70	0	0.07	0.01	0	1.1	0.22	1817	1960	93
3	4.0	225	6.95	1.39	0.95	0.65	0.88	0.60	0	1529	1730	88
4	5.5	245	1.70	6.20	1.10	1.55	1.35	0.60	0.5	1320	1480	89
5	4.5	250	0.06	8.45	0.11	0.44	1.36	7.5	6.6	1217	1350	90
6	5.0	250	0.12	8.30	0.25	3.0	1.34	21.0	10.7	1262	1400	90
7	9.0	215	8.00	0.50	0.27	0.33	0.03	2.6	1.65	1449	1860	78
8	9.0	215	6.80	0.85	0.56	0.65	0.26	2.5	4.10	1342	1730	78
9	9.0	240	0	8.9	0.07	0.65	1.29	7.6	11.4	1252	1350	93

sulfuric acid in terms of millimoles produced from the 11.8 millimoles of cysteic acid used at the beginning. Lactic acid (column 9) is expressed in terms of micromoles. Column 11 expresses the sums of the analytically determined amounts of cysteic acid, taurine, alanine and ammonia calculated in terms of milligrams, for comparison with the total solids as determined by direct weighing (column 12). The figures of column 11 over those of column 12 express in column 13, as percentage, the recoveries of total solids based on this approximate summation. Because of the acidity of the solutions resulting from the runs, either from undecomposed cysteic acid or from sulfuric acid, it was considered valid to include the ammonia. Lactic acid was formed in amounts too small to affect significantly these approximate recovery figures.

The check on analytical results afforded by the weight of total solids recovered is obviously only an approximation. Probably appreciable amounts of unknown and undeterminable material were produced. Moreover, the solids of the evaporated bomb fluids were usually hygrosocpic and any error in the figures of column

12 is undoubtedly in the direction of their being too high. In any case, however, the groups of substances give, when calculated in milligrams, total weights (column 11) averaging 85 to 90 % of the actually determined weights (column 12). The difference represents either analytical errors or the presence of decomposition products other than those determined. The lowest recoveries are found in those runs of longer time.

Recoveries, in terms of millimoles of the 11.8 mM. cysteic acid added, were also calculated from the sums of the taurine, alanine, ammonia, and unchanged cysteic acid. On the assumption that any sulfuric acid formed was the result of direct hydrolysis of cysteic acid and was therefore equivalent to the alanine formed, the latter only was included in this summation of nitrogenous products. Recoveries, calculated in this way, ranged from 76 to 99 %, the highest referring to Run 6 and the lowest to Run 8. As indicated above, the lowest recoveries were usually found in the 9-hour runs.

From the results of runs 1 to 6 it is possible to judge the effect of temperature on the yield of taurine since these runs were of approximately the same length of time. Obviously, little or no decarboxylation takes place at temperatures below 220°C. but mounts rapidly from this point until at 240°C. over a 70 % conversion to taurine can be obtained. Thus, we find that the rate of taurine formation is extraordinarily sensitive to temperature changes within the range of 220 to 240°C. At 240° a doubled period of heating (9 hours) produced the highest yield (over 75 %) of taurine. Our findings thus support the conclusion of White and Fishman that decarboxylation proceeds best in a narrow range of temperature but leave unexplained the detrimental effect of temperatures above 250°C. except to suggest that the temperature coefficient for complete cleavage is greater than that for simple decarboxylation. Runs attempted at 270°C. gave evidence that at these temperatures there is a much greater degree of complete fragmentation of the cysteic acid. A sample of taurine kept at 265°C. for 5 hours showed practically no decomposition and gave a quantitative recovery of total solids. Alanine, under the same conditions, underwent complete decomposition. We conclude, therefore, that the taurine, once formed, is stable in aqueous solution at higher temperatures, but that the course of the decomposition of unchanged cysteic acid is much different if these higher temperatures are quickly reached.

The maximum yield of alanine (less than 10 mole per cent) is attained at about the middle of the temperature range studied (190 to 250°C.). Since this maximum appears in the range in which taurine formation is greatly accelerated, the lower yield of alanine above this range may well be due to the decreasing amounts of cysteic acid available for alanine formation as well as to the further decomposition of the latter which causes a decrease in yield to nearly zero per cent above 250°C.

The other products of the fragmentation of these compounds include carbon dioxide, carbon monoxide, nitrogen, sulfur dioxide, hydrogen sulfide, ethylamine, ethyl mercaptan and hydrogen. Their formation can easily distort the proportions of the products which have been determined. While it is logical to assume that the production of alanine is accompanied, mole for mole, with that of sulfuric acid by

simple hydrolysis, such a rough equivalence holds only up to about 245°C.; above that temperature such correspondence is absent and the molar production of sulfate far exceeds that of alanine, indicating a secondary reaction in which alanine is destroyed.

Enzyme Experiments—In conjunction with the above studies we have investigated further the enzymic decarboxylation reported by Blaschko (1942, 1948). Since he used the Warburg technique, measuring only the carbon dioxide evolved, it seemed worth while to repeat these reactions with actual identification of any taurine by means of paper chromatography. Our technique was similar to that of Blaschko. A homogenate was prepared by disintegrating fresh dog liver with its own volume of a pH 7.4 phosphate buffer in a Waring blendor, cooled with ice. After centrifuging, five ml. of this homogenate were placed in a 50 ml. Erlenmeyer flask together with the test solution, the flask swept with nitrogen and stoppered. After incubation for one hour at 37°C., the action was stopped by heating to boiling. The coagulated residue was extracted twice with hot water and the extract filtered. Four flasks were used for each run. Two contained the substrate, l-cysteic acid, in different amounts (0.005 and 0.025 millimoles); the other two flasks served as controls. One of the latter contained the liver homogenate but no substrate and served as a check on the amino acid pattern of metabolizing liver extract under the conditions of the run. The other control flask was an inactive blank containing the enzyme preparation previously killed by heating, along with the substrate in amount equal to the more dilute of the two reaction flasks. This solution served to indicate whether or not the cysteic acid was passing through the whole procedure in amounts large enough to be detected on the chromatogram. It also made possible a comparison between the amount of cysteic acid originally added and that which escaped conversion in the active samples.

In a series of 10 such experiments, using dog's liver prepared by various modifications of Blaschko's method, no evidence was obtained for the conversion of cysteic acid. Otherwise, there was much enzymic activity as shown by differences in intensity among corresponding spots from the active solutions and the controls. In general, autolysis of the liver tissue gave increased amounts of the amino acids already present but there was no evidence of taurine production, even in the smallest detectable amounts.

The above experiments were duplicated with fresh rat liver, mentioned by Blaschko as a rich source of cysteic acid decarboxylase. Four such experiments all gave strongly positive results. The inactivated blanks, containing cysteic acid, gave for the latter, spots of circular forms and high color intensity whereas in the active runs the resulting cysteic acid spot was weak in color and irregular in shape. The positive spots for taurine were quite definite.

Various methods of extracting the enzyme were also used. It was found preferable with rat liver to grind it in a mortar with sand at about 2°C. since the use of the blendor gave a liquid too homogeneous to be readily centrifuged. The desired enzyme appeared in the more soluble portions of the liver proteins and such complete disintegration was therefore of no advantage.

Activity was not inhibited by oxygen or 8-hydroxyquinoline. It was not promoted by adenosine triphosphate in .0005 M. concentration. Studies of the effect of pH suggested maximum activity in the range from 7 to 8. Racemic cysteic acid was incompletely converted under conditions which gave complete conversion of the laevo form, thus confirming Blaschko's finding that the enzyme is stereospecific. The enzyme was completely inactivated by heating for a five minute period on a steam plate. Added taurine was not further changed by active preparations of the enzyme.

Two-dimensional chromatograms were also run on certain samples to produce more complete separation of taurine and glycine. These extended the results of the one-dimensional chromatograms by demonstrating the presence of an appreciable amount of preformed taurine in rat liver and they also confirmed the significant increase in the taurine content of active samples to which cysteic acid had been added. Along with the disappearance of the cysteic acid, the formation of increased amounts of alanine, a finding suggested by some of the results with one-dimensional chromatograms, was confirmed. It is interesting to find indications, in an enzymic system, of the reaction encountered in high temperature decomposition of cysteic acid: the production, probably hydrolytic, of alanine.

The sharp distinction between our results, negative with dog liver and positive with rat liver, is of interest, particularly in view of the findings of Tarver and Schmidt (1942) and also of Blaschko (1942). The former authors obtained much more definitely positive results from dogs than from rats in tracing radioactive sulfur, fed as methionine, to the taurine of the bile. The latter author obtained evidence of carbon dioxide formation from cysteic acid due to decarboxylase from livers of the dog, rat, pig and guinea pig. Sloane-Stanley (1949) reported a sexual difference in the case of rats, with about twice the concentration of cysteic acid decarboxylase in the livers of males as in those of females. Since the dogs we used were all female whereas the rats were chiefly male, it may be possible to reconcile our findings with those of the above investigators.

SUMMARY

1. The application of chromatographic technique to the detection and approximate estimation of taurine and cysteic acid has been studied. It has been found possible to use this technique in following the decarboxylation of cysteic acid to taurine.

2. The conversion of cysteic acid to taurine in water solution has an optimum temperature range of 240 to 250°C. Heating at this temperature for 4 to 9 hours produces a maximum conversion of about 75 moles per cent. In spite of the stability of taurine at much higher temperatures very little is formed above 25°C.

3. A small proportion of cysteic acid is broken down to alanine. The maximum production of alanine (at about 230°C.) is about 10 moles per cent. At lower temperatures more alanine than taurine is produced.

4. A number of minor decomposition products also occur during *in vitro* decarboxylations, especially at the higher temperatures.

5. The enzymic conversion of l-cysteic acid to taurine by water extract of rat liver has been demonstrated. Both taurine and alanine appeared as metabolic products.

6. Attempts to demonstrate the enzymic decarboxylation of cysteic acid by extracts of dog liver gave negative results.

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SOME OBSERVATIONS ON STRUCTURE AND BEHAVIOR IN THE CILIATE *DILEPTUS MONILATUS*

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Of the 17 species of *Dileptus* recognized by Kahl (1931), several of the commoner ones show certain striking similarities of structure and behavior and are therefore difficult to identify readily. For example, *D. monilatus* (= *Amphileptus monilatus* of Stokes, 1886), because of its general shape and proportions, may be mistaken for *D. anser* (O. F. Müller, 1786), though staining reveals that they have quite dissimilar macronuclei. Likewise, *D. monilatus* may be confused with *D. cygnus* (Claparède and Lachmann, 1859), to which it is admittedly closely related. These two species show similarities in macronuclear structure and arrangement of contractile vacuoles, but they differ with respect to the caudal process and length of the proboscis. Of the three, only *anser* has received special study, the contributions of various investigators, among them Prowazek (1904), Visscher (1927), Studitsky (1930), Hayes (1938), and Jones (1951), having treated of its structure, feeding reactions, division, conjugation, encystment, and excystment. Information on *monilatus* is expecially meager, though *cygnus* is somewhat better known, since there is no doubt that the form which Wrzesniowski (1870) studied and described as *D. gigas* var. *grojecensis* was actually *D. cygnus*. With reference to *monilatus*, we have been unable to find in the literature any comprehensive study whatsoever of its structure and general biology. Conn (1905) mentions its occurrence in Connecticut, but his report omits all species descriptions, and his Figure 157 is none too instructive, since it shows a very atypical proboscis. Thus the diagnoses of Stokes (1886) and Kahl (1931, p. 205) remain as the only satisfactory descriptions that we have found. Each is accompanied by a creditable figure. The significant points of Stokes's original description are the following: Proboscis "forming one-fourth of the entire length of the body, the posterior attenuate tail-like part about one-sixth of that length . . .; contractile vesicles small, numerous, in a single series along the dorsal border, but not extending into the posterior attentuation; nucleus moniliform, the nodules small, ovate. . . . Length of body $\frac{1}{35}$ inch" (714μ). Stokes's figure shows a total of ten contractile vacuoles, three of which are in the proboscis. The micronuclei are unmentioned.

Kahl remarks that the specimens which he himself observed differed in certain respects from those of Stokes, *viz.*, they had on the average a longer proboscis, macronuclear segments of greater size, more contractile vacuoles in the proboscis, and a better defined caudal process. Whether these are actually significant differences is questionable, since a certain amount of variation within a species is expected and since Kahl's optical equipment was assuredly superior to that of

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Stokes. The essential features of Kahl's diagnosis follow: Proboscis one-third to nearly two-thirds as long as the trunk; macronucleus "kettenförmig," consisting of 15–22 ovoid parts, each 15–20μ long; 5–10 contractile vacuoles in the proboscis; posterior end terminating in a well-defined, long, thin caudal process, not tapering gradually to a point as Stokes's figure shows it; micronuclei spherical, 3–5μ in diameter, in number about equal to the macronuclear segments; length 500–900μ.

The present study affords some further observations on the structure and behavior of *monilatus*, with incidental mention of *cygnus*, and it directs attention to several rather easily demonstrable peculiarities of structure and behavior which, it is believed, will be of aid in the ready identification of the three aforementioned species.

MATERIAL AND METHODS

Our specimens of *monilatus* came from two sources: one group was collected near Chapel Hill, a second was kindly supplied by Mr. W. R. Pendergrass of the Carolina Biological Supply Co., Elon College, North Carolina. No significant differences could be noted between individuals of the two groups. Both *anser* and *cygnus* were collected near Chapel Hill.

For special study, specimens of *monilatus* were fixed in Schaudinn's fluid with added acetic acid, and stained by one of the following: Heidenhain's iron hematoxylin; carmalum and Lyons blue; Mallory's phosphotungstic hematoxylin; the Feulgen reaction.

OBSERVATIONS

Although *monilatus* swims in the manner of *anser* and *cygnus*, rotating on its longitudinal axis, it is primarily a bottom-dwelling species. Usually it is found moving slowly through detritus and masses of zooglea, probing constantly with its proboscis. This probing action is not seen in *anser*, which is essentially an active, swimming species, though Canella (1951, p. 157) points out that it does not swim incessantly ("unaufhörlich"), as Wrzesniowski (1870) states. (It is generally agreed that Wrzesniowski's *D. gigas* var. *varsaviensis* was *D. anser*.) The probing action also occurs in *cygnus*, which like *monilatus* is bottom-dwelling and somewhat sluggish—"träge," as both Wrzesniowski and Kahl remark—but the proboscis of *cygnus* is exceptionally long, flexible, and extensible. Kahl (p. 205) states that it is distinctly longer than the trunk, a fact confirmed by Canella's Figure XII and by our own observations. The proboscis of *monilatus* is relatively shorter, varying from one-third to one-half as long as the trunk in our specimens (Fig. 1).

The trunk of *monilatus* ends in a remarkably long caudal process (Figs. 1 and 6, TA). It is heavily ciliated and in some individuals it is almost as long as the proboscis. Its great length is a distinguishing feature of *monilatus*; in *cygnus* a caudal process is absent, and the posterior end tapers abruptly to a point; in *anser* the process is short, constituting little more than one-sixth of the trunk length, and it usually bears a sticky, trailing caudal thread, which serves at times to anchor the animal to the substratum (Jones, 1951, p. 220; Canella, 1951, p. 156).

In life the body of *monilatus* is remarkably transparent, thus permitting the observation of various internal structures. For example, the beaded macronucleus is plainly visible, though the micronuclei cannot be seen. The macronucleus in *cygnus* is similarly beaded but cannot as a rule be seen in the living condition ("schwer erkennbar," according to Kahl), nor can it be seen in the living *anser*, in which it takes the form of many generally dispersed components. Likewise, the contractile vacuoles, about 20 in number, are easily seen in the living *monilatus*. They occur as a single row which extends from the tip of the proboscis posteriorly along the dorsal margin to the region of the cytopyge, which is found at the base of the caudal process. The specimen shown in Figure 1 had 19 contractile vacuoles in all, eight in the proboscis, meaning anterior to the cytostome. In *cygnus* the arrangement and distribution of the vacuoles are much as in *monilatus*. In *anser* their arrangement is fundamentally the same, but usually no more than one or two occur anterior to the cytostome.

The proboscis of *monilatus* bears several rows of trichocysts medially along its entire ventral or oral surface. The rows are bordered laterally by the long cilia of the feeding groove (Fig. 3, TR and FG). Trichocysts cannot be seen well in the living animal, but upon staining with iron hematoxylin they may be demonstrated satisfactorily either *in situ* (Fig. 3) or discharged (Fig. 2). The discharged trichocyst resembles in shape a long, tapered, flattened, homogeneous blade, having a length of 6μ and a maximum width of slightly less than 0.5μ. Discharged trichocysts often appear twisted, and in general they do not stain as well as when *in situ*.

The mouth apparatus of *monilatus* consists of a cytostome and pharyngeal basket. The cytostome (Figs. 3 and 4, CS) is located at the posterior end of the feeding groove; it is a shallow depression embraced by the outer ends of the trichites which form the pharyngeal basket (Figs. 1, 3, and 4; PH). The basket is a relatively large structure which is funnel-shaped and slightly arched, with its inner end directed postero-dorsally. It is clearly visible in the living animal.

It is well known that *anser* is an omnivorous predator, feeding principally on other ciliates, but ingesting flagellates and rotifers as well (Visscher, 1923). It is commonly cultured on the oligotrich *Halteria grandinella*. The food habits of

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FIGURES 1–6. The holotrichous ciliate *Dileptus monilatus* (Stokes, 1886)

FIG. 1. Whole mount of adult, to show general structure. Body cilia omitted, except on feeding groove. Schaudinn-iron hematoxylin. Camera lucida.

FIG. 2. Trichocysts discharged upon fixation in Schaudinn's fluid. Iron hematoxylin.

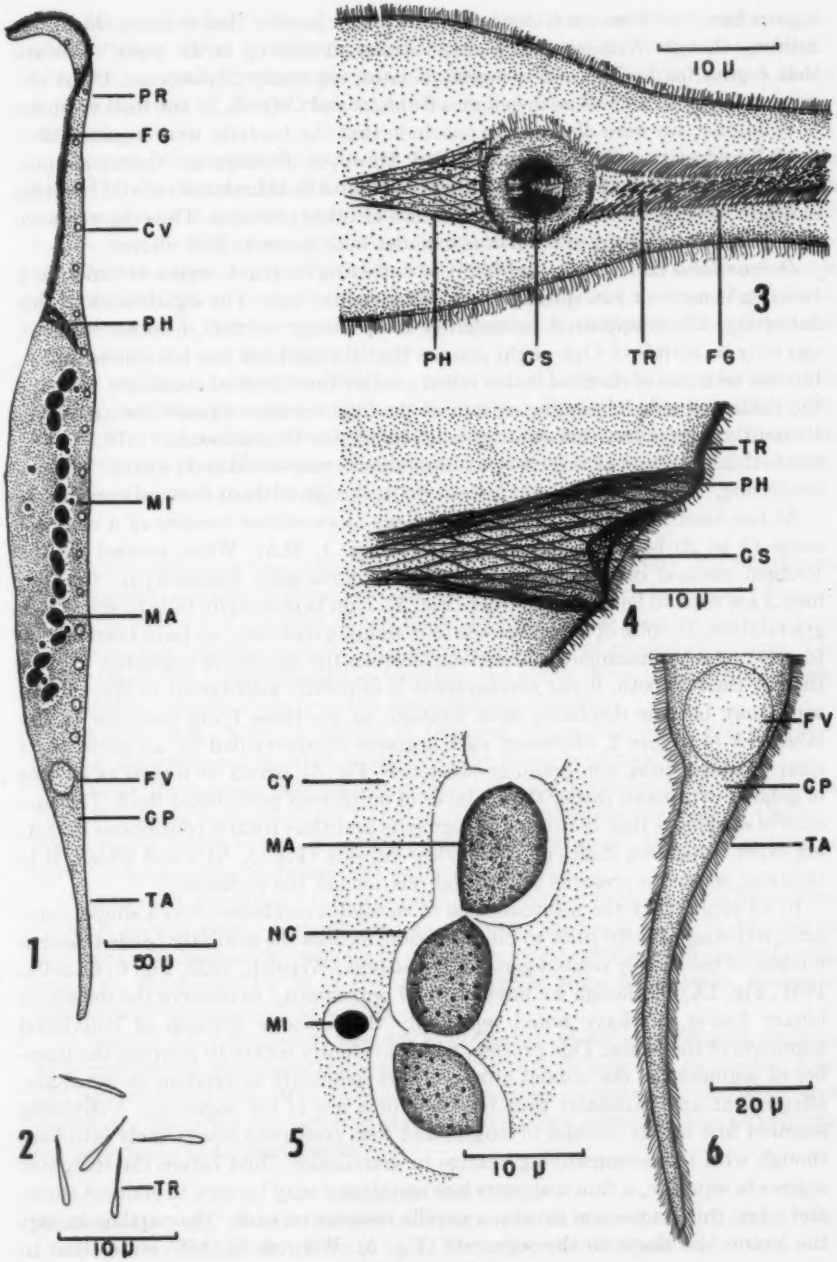
FIG. 3. Ventral view of cytostome, showing ciliated feeding groove and trichocysts *in situ*. Schaudinn-iron hematoxylin. Camera lucida.

FIG. 4. Lateral view of cytostome, showing pharyngeal basket. Schaudinn-iron hematoxylin. Camera lucida.

FIG. 5. Three segments of the moniliform macronucleus, and an adjacent micronucleus. Schaudinn; Feulgen reaction. Camera lucida.

FIG. 6. The long, well-defined caudal process. Schaudinn-iron hematoxylin. Camera lucida.

CP, position of cytopyge; CS, cytostome; CV, contractile vacuole; CY, cytoplasm; FG, feeding groove; FV, food vacuole; MA, macronucleus; MI, micronucleus; NC, nuclear capsule; PH, pharyngeal basket; PR, proboscis; TA, caudal process; TR, trichocysts.


PR
FG
CV
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FV
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50 μ
1
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TR
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CS
10 μ
4
CY
MA
NC
MI
10 μ
5
FV
CP
TA
20 μ
6

FIGURES 1–6.

cygnus have not been carefully studied. Canella implies that it feeds chiefly on rotifers, though Wrzesniowski's observations on feeding in "*D. gigas*" indicate that *cygnus*, as well as *anser*, ingests ciliates, especially *Stylonychia*. Upon observing bacteria, as well as the ciliates *Euplotes* and *Colpoda*, in the food vacuoles of *monilatus*, we were disposed to conclude that the bacteria were ingested incidentally when the ciliates were engulfed. However, Pendergrass (personal communication) reports that he has cultured *monilatus* in abundance on wild bacteria in dilute wheat-seed infusion in the absence of other protozoa. Thus the evidence indicates that the food of *monilatus* includes both bacteria and ciliates.

D. monilatus has the peculiar habit of flattening its trunk region at times, and twisting it more or less spirally on the longitudinal axis. The significance of this flattening, which appeared consistently in seemingly normal, healthy cultures, can only be surmised. One might assume that the medium was too concentrated, but the addition of distilled water failed to alter the flattened condition. Perhaps the flattening aids in pushing certain of the food vacuoles toward the cytopyge. Evidently *cygnus* has this same peculiar habit, for Wrzesniowski (1870, p. 508) states that the trunk of this species is sometimes compressed and twisted "schraubenförmig," while the proboscis lashes about, though without forward movement.

As has been mentioned, the macronucleus of *monilatus* consists of a chain of some 15 to 20 bead-like, ovoid segments (Fig. 1, MA). When stained by the Feulgen method or with carmalum or phosphotungstic hematoxylin, the segments are seen to be finely granular (Fig. 5). Iron hematoxylin fails to show such granulation. In spite of the use of the four staining methods, we have been unable to observe any permanent connections between the successive segments. Nevertheless, their smooth, linear arrangement is faithfully maintained in life, though some may become displaced upon fixation, as are those lying posterior to the label MA of Figure 1. However, each segment is surrounded by an envelope of clear, homogeneous, non-granular substance (Fig. 5), which we regard as an area of gelated cytoplasm rather than a layer of indifferent perinuclear fluid. The successive envelopes fuse between the segments and thus form a continuous investing layer, which we shall call the nuclear capsule (Fig. 5, NC) and which, it is believed, serves to preserve the spatial relations of the segments.

In all probability the macronucleus of *monilatus* condenses into a single, compact, rod-shaped body prior to binary fission, as does the similarly beaded macronucleus of the closely related genus *Paradileptus* (Wenrich, 1929, Fig. 6; Canella, 1951, Fig. IX). Although we have not had opportunity to observe the details of binary fission, we have noted repeatedly the amitotic division of individual segments of the chain. This process, which evidently serves to increase the number of segments as the animal grows, occurs seemingly at random in the chain, affecting at any particular time only one or a few of the segments. A dividing segment first nearly doubles in length, and then constricts transversely into two, though with no accompanying change in granulation. Just before the daughter segments separate, a thin macronuclear membrane may be seen to connect them, and when this connection breaks, a papilla remains on each. The papillae impart the lemon-like shape to the segments (Fig. 5). Wenrich (p. 355) states that in

Paradileptus conicus "there is good evidence that the number [of segments] increases after fission by elongation and constriction of the several segments." Evidently a similar process occurs in *P. robustus*, for Wenrich's Figure 5 shows a macronucleus of 15 discrete segments, two of which are elongated and constricted.

The number of micronuclei in our specimens of *monilatus* varied from five to nine. Usually they are found alongside the macronuclear capsule (Fig. 5), though some may be scattered at random in the cytoplasm (Fig. 1). They stain intensely by the four methods used, and in such preparations each is surrounded by a distinct hyaline area (Fig. 5). Actually, it is not clear whether this area represents a fluid-filled space between the micronucleus proper and the cytoplasm, or a space between a thin micronuclear membrane and an endosome. The division of the micronuclei was not observed.

It is our opinion that *monilatus* is commoner than is assumed and that it is usually mistaken for *anser* in general collections. For example, Bütschli (1889, Pl. 59, Fig. 4a) shows a specimen which he regards as a form of *anser*. Since the figure shows a moniliform macronucleus and a proboscis half as long as the trunk, the specimen was undoubtedly *monilatus*.

SUMMARY

Dileptus monilatus feeds upon bacteria and upon other ciliates. Its moniliform macronucleus consists of some 15 to 20 ovoid segments which lack morphological connections one with another. Their linear arrangement is maintained by a hyaline, investing "nuclear capsule," interpreted as a layer of gelated cytoplasm. The segments divide independently to lengthen the chain. Five to nine micronuclei are present, usually lying near the macronuclear capsule.

The three fairly common species of *Dileptus*—*anser*, *monilatus*, and *cygnus*—may be distinguished one from another in the living condition as follows.

D. anser: Swimming persistently, usually trailing a caudal thread; proboscis ⅓ to ½ as long as trunk; only 1 or 2 (rarely 3) contractile vacuoles in the proboscis just anterior to the cytostome; caudal process short, about ⅙ as long as trunk; macronucleus dispersed and indistinguishable.

D. monilatus: Essentially bottom-dwelling; probing constantly with its proboscis as it moves slowly through bottom detritus; proboscis ⅓ to ⅔ as long as trunk; 5 to 10 contractile vacuoles in proboscis; caudal process well-defined, long, and thin, accounting for ⅓ of the trunk length in some individuals; macronucleus moniliform and clearly visible.

D. cygnus: Like *monilatus*, essentially bottom-dwelling and sluggish, probing with proboscis in *monilatus* fashion; proboscis very flexible and extensible, usually longer than trunk; 10 to 15 contractile vacuoles in proboscis; caudal process absent, the posterior end tapering abruptly to a point; macronucleus moniliform but practically indistinguishable.

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SALAMANDERS OF PITT COUNTY, NORTH CAROLINA

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Pitt County is in the middle part of the coastal plain of North Carolina, and drained largely by the Tar River which crosses it, although in the west and southwest the streams are tributaries of the Neuse. Eastward the county approaches tidewater near Washington, in Beaufort County. The streams are bordered by cypress swamps and mixed hardwoods, the latter extending up low slopes to the general level of the surrounding land. This is partly occupied by pine and oak forest, but mostly under cultivation. The soil is sandy loam, its organic content highest under swamp forest. There is no exposure of rock.

Published records of salamanders in Pitt County are two by Brimley (1939–43): *Plethodon c. cinereus* and *Eurycea longicauda guttolineata;* seven by Robertson and Tyson (1950), including the second above but not the first. The additional species are *Amphiuma means, Desmognathus fuscus auriculatus, Plethodon g. glutinosus, Stereochilus marginatus, Eurycea bislineata cirrigera, Manculus quadridigitatus.* Two localities in the county are represented in Robertson and Tyson's report, Chicod Creek near Grimesland (east) and Little Contentnea Creek (west). The total list previously recorded, then, is eight species.

During the past three years, on numerous collecting trips alone and with students from East Carolina College, Greenville, the writer has obtained all the above species and six more, not previously reported. Information has been accumulated on the habitats and relative abundance of the various forms, but the county list may eventually be increased by two or three more, notably *Siren lacertina* and *Ambystoma maculatum.* I should like particularly to acknowledge the aid of Walter C. Biggs, John B. Funderburg and Claude H. McAllister in connection with *Amphiuma means, Ambystoma t. tigrinum, Plethodon c. cinereus,* and *Stereochilus marginatus,* which otherwise might not yet be represented by local specimens. The salamanders are in the collections of East Carolina College and the author. For the commoner species it does not seem necessary here to record exact sites and dates, but that is done for the more significant finds.

Necturus lewisi Brimley. Lewis's Mudpuppy

One specimen (199 mm.) of this sandy brown, dark-spotted species was taken from the Tar River at Greenville, February 13, 1951. Brimley (1924) described it as a subspecies of *Necturus maculosus,* from running water of the Neuse River near Raleigh, and reported it also from Kinston, Chapel Hill, and Tarboro (Tar River). Bishop (1943) treats it as a species. Judging from the small amount of information now available, it occurs in rivers where the bottom is muddy or sandy, and the water is moving.

Necturus punctatus (Gibbes). Southern Mudpuppy

Larvae and adults of this smaller, darker species have been collected in a number of localities, most frequently along a small stream three to five miles east of

Greenville, tributary to the Tar River. This extends the known range northward as *N. punctatus* has not been reported from the Tar drainage (see Bishop, 1943). The dark, uniform color of the upper parts, and the situations from which we have taken it by dipnets, suggest that it occupies a different ecological niche from that of *N. lewisi*, namely the small shady creeks, whose muddy bottoms carry accumulations of dead leaves.

Amphiuma means (Garden). Congo Eel

Robertson and Tyson (1950) reported one specimen found ten miles west of Greenville in a marshy pasture. We obtained four small ones by dipnet from a muddy stream in a cypress swamp five miles southeast of Greenville.

Triturus viridescens dorsalis (Harlan). Broken-stripe Newt

Two males and a female, in breeding condition, were taken March 10, 1941, at "Golf Course Pond," near Greenville. The newt seems to be scarce here and of quite limited distribution. Of these specimens one shows the stripes characteristic of *dorsalis*, but the others do not, which implies that Pitt County lies in the zone of intergradation with *T. v. viridescens*. Gray (1941) lists newts from the Duke Forest, near Durham, as *T. v. viridescens* only.

Ambystoma opacum (Gravenhorst). Marbled Salamander

Although not particularly abundant, this species has been found under logs on both sides of the Tar River, and in woods adjoining the college. Larvae, about half grown, were collected January 31, 1953, in a springfed marsh 6 miles southeast of Greenville by Miss Lucile Rice and Dr. O. Christine Wilton.

Ambystoma tigrinum tigrinum (Green). Tiger Salamander

The only specimen was an adult brought in by a high school student from the outskirts of Greenville in September, 1952, which unfortunately we were not able to retain.

Desmognathus fuscus auriculatus (Holbrook). Southern Dusky Salamander

This coastal plain species is abundant and widely distributed along watercourses throughout the region. Locally it outnumbers all other salamanders combined. Larvae can be taken by dipnet in pools and running streams in the woods. Neill and Rose (1949) first reported a nest with a female attending six eggs, near Augusta, Georgia, June 16, 1948, remarking that "recently hatched larvae and females with ovarian eggs had been collected . . . a few days previously; evidently the breeding season is an extended one." Robertson and Tyson (1950) described six nests containing fourteen to twenty eggs in rotted logs and stumps near Grimesland, Pitt County, N. C., September 5, 1948. On a field trip to a cypress swamp five miles southeast of Greenville in late October, 1952, we found a female under a log in wet mud, with two unhatched eggs and fifteen young. Finally, January 21, 1953, Walter Biggs and Claude McAllister found a female under a log in a similar situation just south of the college, with twenty-

two young; some were four or five inches from her, but most were in a cluster in contact with her. It appears that the breeding season is indeed an extended one, and that after hatching the young, usually 15–16 mm. in length, may remain with the female for weeks, if not months, before taking to the water. Their color in this stage is nearly black, the usual spotted pattern and browner tone appearing later, in the aquatic phase. It also seems that the aquatic larvae represent a growing stage of several months' duration, transforming at 35–42 mm. in January and February (so far as our observations go), but more work will be necessary before we can be sure of the normal sequence of events.

This may be the place to comment upon Grobman's (1950) study of the southern races of *Desmognathus fuscus*. His work was based upon a large amount of material, more, apparently, than was available to Bishop (1943), and from it a quite different map of the distribution of *D. f. auriculatus* and *D. f. brimleyorum* resulted. Bishop had shown *brimleyorum* restricted primarily to Arkansas, from which it was originally described, while *auriculatus* ranged from Louisiana east along the coast to northern Florida, and north to the Dismal Swamp, in Virginia. Practically the only distinctive key character for their separation was the light, uniform pigmentation of the belly in *brimleyorum*, and the light or dark but mottled belly of *auriculatus*. This is shown clearly in the photographs, and we may note here that by this character all Pitt County specimens are *auriculatus*. Other characters of each appear, from careful reading of the descriptions, to be alike or sufficiently overlapping to be unreliable for separation.

Sanders and Smith (1949) reported a single specimen of each from Hardin County, Texas, but stated that other specimens have been collected and are available; they were not able to say whether intergradation occurs, or just how distinct the two races are.

Grobman, not depending entirely upon preserved material, compared live specimens from the Ozarks, from New Orleans, and from Gainesville, Florida. Those from the first two places he described as "brown or light gray" and the Florida salamanders as "very dark brown to jet black." By exposing a series of each to continuous light for ten days and another series to continuous darkness, he was able to show that the light exposure resulted in lightening the color of both the brown and the black types. But he says that the two were still distinguishable from each other, evidently on account of a "residual amount of color difference that is presumably due to the difference in genetic make-up."

He notes that the "brown" race is *brimleyorum*, but makes no further statement of a difference between the two than that quoted above, which scarcely seems to satisfy the needs of subspecific definition. Nevertheless, "looking through collections of preserved material," he determines the black ones as *auriculatus* and the browns (and grays) as *brimleyorum*. Supposedly this is based upon that "residual amount of color difference" which is the only real character mentioned, and which was found in the experimental series. *Brimleyorum* is extended east to the Dismal Swamp, and *auriculatus* restricted to "black soil" from Mississippi to Florida and southern South Carolina.

To the present writer it seems that while one may so map the distribution of

brown-gray and dark brown to black salamanders, it is a different matter to tag them as subspecies, rearranged over an enormous area, without indicating any other workable characters. On the somewhat subjective color basis, Grobman places all coastal plain specimens from North Carolina as *brimleyorum*, although in both living and preserved specimens we find variation from light brown to very dark brown if not jet black. The ventral pattern, as mentioned, is not that of the *brimleyorum* from the Ozarks, at least. Thus I am using the name *auriculatus* since, in Bishop's sense, the animal appears to be recognizable, and because, from Grobman's study, I am not yet clear as to the degree of darkness or lightness which would separate them, or the validity of color intensity alone as a character in this case.

Plethodon cinereus cinereus (Green). Red-backed Salamander

Although listed from Pitt County by Brimley (1939–43), this is not a common species and appears to have extremely limited distribution in eastern North Carolina. Only four specimens have been taken since Brimley's record, from beneath logs in one small spot just south of the college, in February, March and April. The site is mixed beech and oak forest bordering on, but a few feet higher than, a swamp.

Plethodon glutinosus glutinosus (Green). Slimy Salamander

Six specimens from slopes above a cypress swamp ten miles west of Greenville were reported by Robertson and Tyson (1950). Many other similar locations have given us further material, in or under logs. Some specimens come from wet situations shared with *D. f. auriculatus*, but the majority are on better-drained slopes. The smallest specimens (both from Greenville) are: 24 mm., February 8, 1953; 29 mm., February 20, 1951.

Pseudotriton montanus montanus Baird. Baird's Red Salamander

Two adults taken in a springy spot in woods six miles southeast of Greenville, January 18, 1952, are the only ones so far found in Pitt County. As to its range, Bishop's (1943) map appears to need revising, because Brimley (1939–43) had already recorded it from other coastal plain counties, including Carteret, New Hanover and Brunswick, so that it is not limited to the piedmont and western section.

Eurycea bislineata cirrigera (Green). Southern Two-lined Salamander

This salamander is widespread and common, but seldom found far from water, often under damp logs on slopes. Several times we have taken it by dipnets in running streams during the winter. Such individuals, if adult, were females containing eggs; aquatic larvae are occasionally found.

Eurycea longicauda guttolineata (Holbrook). Three-lined Salamander

Robertson and Tyson (1950) reported a single specimen from ten miles west of Greenville, September 4. We took two from inside a large rotted cypress stump

and another from beneath a log in a cypress swamp five miles southeast of Greenville, March 1, 1951. Other visits to the same spot have been unsuccessful.

Manculus quadridigitatus quadridigitatus (Holbrook). Dwarf Salamander

Few North Carolina records have been published for this species (Wake, Lenoir, Duplin and Columbus counties, by Brimley, 1939–43, and one for Pitt county, by Robertson and Tyson, 1950). But it is not uncommon in the vicinity of Greenville, occupying the same situations as the Two-lined Salamander, to which it bears a close resemblance as if it were a pygmy relative. Our specimens are adults, taken under logs, usually away from the wetter ground.

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PROTECTIVE BEHAVIOR AND PHOTIC ORIENTATION IN HATCHLING SNAPPING TURTLES, *CHELYDRA SERPENTINA SERPENTINA* (LINNÉ), IN AN AQUATIC ENVIRONMENT

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In several recent reviews of North American chelonians (Pope, 1939 and 1944; Carr, 1952) the natural history of the snapping turtle has received considerable atter tion. No information, however, has been published on the protective behavior and photic orientation of the hatchlings in an aquatic environment. Differences in the behavior of adults and hatchlings are related to differences in the environmental stresses to which they are exposed. For example, in the case of their natural enemies, Hamilton (1940) mentions a number of hatchling predators, and Pope (1944) points out that with the single exception of man the ". . . mature turtles seem to have no . . . important enemy, but the very young are eaten by crows, hawks, mink, raccoons, large fishes, and even bullfrogs."

The activity patterns of hatchlings result from an interaction between biologic demands and protection requirements as influenced by photic stimulation, which is correlated with the characteristics and efficiency of the optic apparatus. The snapper is reported to be "light-shy," and a typical nocturnal retina containing a marked predominance of rods over cones would be expected. Turtles, however, are conspicuously exceptional in having only a few rods scattered over almost pure cone retinas. The relative number of rods to cones is higher in the snapper than in many other turtle species, suggesting a stronger nocturnal vision, and this is associated with a more pronounced negative photic orientation. As Walls (1942) points out, the explanation of the reversal in the usual rod-cone ratio lies in the fact that the lens of a turtle always projects through the pupil, letting the iris get a grip on it during accommodation, thus the pupil can close but little if at all. Turtles obtain "immunity from dazzlement by eliminating nearly all of their rods, though they might perhaps have kept a well-balanced duplex retina if they had also retained efficient photomechanical changes." The poor development of the average chelonian chorioid strongly suggests that the metabolic requirements of the retina are relatively low, and that turtles are thus under some handicap in seeing under dim underwater conditions. Walls (ibid.) adds that bottom forms such as the snapper "hunt chiefly by touch and smell." From tank observations it is apparent that vision plays a predominant role in food detection. Meat placed in the water near a snapper, but out of its field of vision, frequently fails to arouse the snapper's interest in foraging, while a similar piece of meat moving in its field of vision receives prompt attention. Partial compensation for their poor photomechanical structure is found in the facts that turtles can attain perfect focus whether the head is submerged or above water, and that *Chelydra* have the greatest field of binocular vision of any turtles investigated by

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Walls (ibid.). This is a marked asset since, in feeding, "the snapper strikes its prey like a snake, and thus has special need of good distance-judgment which binocularity confers."

The activity patterns of hatchlings are also related to the effectiveness of their deceptive resemblance to their surroundings. As pointed out by the author (Wood, 1951) in the case of the two-lined salamander, *Eurycea bislineata b.* × *cirrigera*, behavior is related to degree of concealment. The hatchling obtains survival advantage from the similarity between the appearance of its rough carapace and pieces of water-logged bark or lumps of mud in its usual habitat. During the precarious period in the hatchling's life, when predatory enemies are a risk, periods of "sunning" on exposed banks would be incompatible with long survival, and the young turtles are more aquatic than adults. Along streams of the coastal plain in Virginia the adults are frequently seen on exposed banks and mud bars in warm weather, but hatchlings are rarely observed.

Many types of aquatic environment meet the requirements of snapping turtles. According to Pope (1944), snappers may be expected in "any permanent body of water large enough to support an association of aquatic plants, but . . . (show) a decided preference for sluggish water with a mucky bottom and profuse vegetation." Carr (1952) adds that snappers occur more frequently "in bodies of water with soft, muddy banks or bottom (in which they may bury themselves in hibernation or possibly lie in ambush for the animals that form a part of their diet)." In Old Channel, an abandoned section of canal in Dayton, Ohio, eight-months old juvenile *Chelydra s. serpentina* and *Sternotherus odoratus* were found in May and June in both 1940 and 1941. They were buried in the surface layer of the mucky bottom of shallows at the margin of the pond, in water from one to three inches deep which was covered by a heavy layer of duck weed. Adults of these species are present in the same habitat, and in the case of snappers, they are found in deeper water.

To determine the photic orientation and protective behavior of hatchling in an aquatic habitat with a non-concealing background, the author studied the distribution of sixty specimens in the tank in Fig. 1-A. These hatchlings were not fed after emerging from eggs, and at the time of the observations (Oct. 16–24, 1950) most of them still showed the bulge of an undigested yolk mass in the vicinity of the umbilicus. The bed of the tank consisted of a two-inch layer of white brook sand covered by a two-inch layer of brook water. Illumination in the light zone consisted of a 100 watt frosted bulb located 33 inches above the water surface, and the illumination in the twilight and dark zones resulted from the scatter of light from the illuminated area. The pattern of light intensities in the three zones (Fig. 1-B) was measured with a G.E. meter, type DW-58, with an A.S.A. scale. During the observations the water temperatures in the tank ranged from 17°C. to 19°C.

For the initial observation, 20 hatchlings were distributed at random in each of the three zones. Following a period of four hours, during which the hatchlings distributed themselves according to photic stimulations and those of objects moving in their field of vision, the gates between the zones were lowered. The

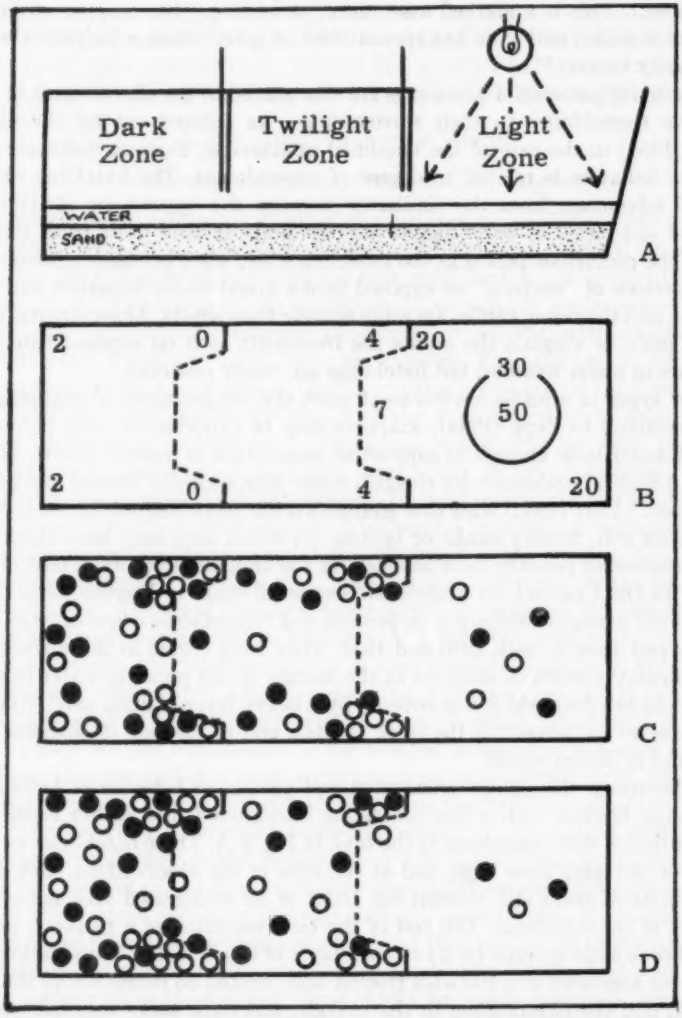

FIG. 1. A. A cross-sectional diagram of the tank used to test photic orientation in hatchling *Chelydra serpentina*. B. A water-surface diagram of the tank showing the distribution of light intensity as determined with a G.E. Meter (type DW-58, A.S.A. scale). C. A diagram of the average distribution of hatchlings prior to feeding. D. A diagram of the average distribution of hatchlings following feeding. In diagrams C and D the black spots represent buried hatchlings, and the circles denote specimens on the sand surface.

positions of the hatchlings on the surface of the sand were charted, and the specimens were removed from the tank. The sand bed was then explored for buried

specimens which were exhumed as their distribution was recorded. Following this the sand bed was leveled and the hatchlings were again distributed in the tank zones as in the initial observation. This procedure was repeated 14 times, with a time lapse of from four to eight hours between observations, and a total of 840 hatchling-distribution observations was thus obtained. Stier (1926) found that the photic responses of newts may be reversed by feeding, that starved newts are negatively phototropic, while well-fed ones are indifferent or positive in response to light stimuli. To determine the effect of feeding on the behavior and photic orientation of the hatchlings, a meal of raw ground beef and fish was provided in another tank. The feeding was voracious, and the hatchlings were then distributed to the tank zones as described previously. A series of seven observations was made, providing a total of 420 hatchling-distribution observations

TABLE I

Distribution of hatchlings prior to feeding

	ON SURFACE	BURIED	TOTAL
Mean number of specimens	37	23	60
Light zone	15.4%	5.4%	11.7%
Twilight zone	31.8%	40.3%	35.0%
Dark zone	52.8%	54.3%	53.3%

TABLE II

Distribution of hatchlings after feeding

	ON SURFACE	BURIED	TOTAL
Mean number of specimens	29	31	60
Light zone	5.0%	6.5%	5.7%
Twilight zone	30.0%	34.5%	31.8%
Dark zone	65.0%	59.0%	62.5%

made shortly after a feeding. The average distribution of surface and buried specimens before and after feeding is shown in Figure 1, C and D. Data on this distribution are compiled in Tables I and II.

The hatchlings prior to feeding were not in a condition of starvation since inanition had not severely depleted their fat supply. However, some changes were noted in their distribution and behavior following feeding. Prior to feeding more hatchlings were noted on the surface, and more were in the light zone. Negative photic orientation and burying activity seemed to increase following feeding, as more hatchlings retreated from sight and light.

The complement of hatchlings in each light zone was characterized by a repeated distribution pattern. Random scatter was found only in the brightest zone, and here the buried specimens were frequently entirely beneath the surface of the sand. In the twilight zone the majority of surface specimens were congregated behind the narrow posts supporting the gate, and occasionally were piled

up two or three deep. This area had the advantage of permitting the hatchlings to remain in a dimly lighted area adjacent to a well illuminated one where objects moving in their field of vision could be readily observed. The buried specimens in the twilight zone were scattered at random over the area, and usually had their heads and necks exposed. The distribution pattern in the dark zone was in all respects like that in the twilight zone except that the presence of greater numbers of specimens resulted in more crowding behind the gate posts, and more specimens scattered over the open area. There appeared to be a greater concentration of specimens along the rear margin of the tank where illumination registered only two A.S.A. units.

The behavior of hatchlings in burying themselves was observed many times. Hatchlings use their hind feet to anchor themselves in the loose sand, and excavate a shallow depression with their front feet. They then retract their heads, and slowly crawl down into their depression, digging their way under the surface as they crawl. Some burrowed as deep as two inches, but most moved just beneath the surface of the sand. After a subsurface crawl of two or three inches the hatchlings would stop, and after a variable period, would protrude their heads and necks up through the sand. It is of some interest to note that no hatchlings were observed attempting to dig their way under the sand with their hind feet since it is known (Carr, 1952) that females scoop out their nests using their hind feet in excavation. No mention is made by Carr of any females digging their nests with their front feet.

SUMMARY

1. Hatchling *Chelydra s. serpentina* show marked negative photic orientation. The orientation is selective for areas of minimum light intensity from which better illuminated zones can be observed.

2. Following feeding, hatchlings show an increased tendency to retreat from light and sight, by negative photic orientation attained by greater dispersal to darker areas or by burial in the sand.

3. Hatchlings burying themselves in the illuminated zone are frequently entirely beneath the surface, while those in the twilight and dark zones usually have their heads and necks protruding from the sand.

4. Hatchlings bury themselves by digging with their fore feet, the hind feet being used only to anchor the specimen during the excavation process, or to aid in propelling the specimen beneath the sand.

ACKNOWLEDGMENTS

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SOME OBSERVATIONS ON THE LAND SNAILS IN THE DUKE FOREST

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The Duke Forest covers an area of 7646 acres (nearly 12 square miles) and has elevations of 280 to 750 feet above sea level. It contains typical forest habitats in which pine tree stands are 120 years old and climax oak-hickory stands contain trees that are more than 200 years old. Several small streams run through the forest.

Preliminary observations indicated that there were few or no snails in pine litter or under pine logs. Between November 9, 1950 and November 25, 1952 observations were limited to stands of broad leaved trees. Eighty-two collections were made on different days, 41 at high altitudes that alternated with 41 at low altitudes near streams. No collections were made between May 10 and July 18, 1951 and April 14 and June 17, 1952, as the writer was then absent from the vicinity.

The procedure followed each day when weather was favorable was to turn over logs for an hour and then sort litter from the same general area on a small white cloth three times. The cloth measured 41 x 30 inches. Litter from around this, up to a distance of about 16 inches, was piled on the cloth. The litter was then carefully sorted over and any snails found were saved. An area of about 54 square feet was then covered each time collections were made, as three sortings were made each day.

During the latter part of the work, records were made of temperatures and hydrogen-ion concentrations. Temperature readings were made at a depth of one inch in the soil. Forty-nine of these ranged from 4°C. in November, February and March to 22°C. in August; average 11.5°C. The fifty-eight pH readings made from material at the surface of the soil beneath the litter ranged from 5.0 in August, September and October to 7–7.5 in January and November, and more than half the readings at all seasons were 6.0 and the average of all was 6.2.

Snails were identified from Pilsbry's (1939–1948) Land Mollusca of North America. Representative specimens were sent to Dr. Henry van der Schalie, Museum of Zoology, University of Michigan, who checked the identifications. For these grateful acknowledgment is made. The twenty-one species found are shown in Table I. The three species that were found to occur in largest numbers were of intermediate size. The numbers collected at high and at low altitudes are segregated in the table as empty shells and living snails.

Somewhat fewer than two-thirds of the snails were found at high levels. Thirteen species and subspecies were more common at high levels, seven at low. Three species were approximately equal in numbers at both levels; these were *Anguispira alternata palustris*, *Philomyces carolinensis flexuolaris*, and *Zonitoides arboreus* (Table I). Living snails of one species, *Stenotrema hirsutum*, were more

common than empty shells at high levels. This was perhaps due in part to the fact that limestone outcrops were more common there. Four uncommon species and subspecies were found only at low levels: *Helcodiscus parallelus, Triodopsis albolabris, Triodopsis notata,* and *Ventridens intertextus eutropis.* Two uncommon ones were found only at high levels: *Euconulus chersinus* and *Strobilops aenea.*

TABLE I

Distribution of land snails in the Duke Forest

	HIGH LEVELS		LOW LEVELS		TOTAL
	Shell	Living	Shell	Living	
Anguispira alternata palustris (Say)	4		3	2	9
Euconulus chersinus (Say)	1				1
Haplotrema concavum (Say)	174	28	115	27	344
Helcodiscus parallelus (Say)			2		2
Mesodon thyroidus (Say)	28	13	37	13	91
Mesodon thyroidus bucculenta Gould	11	4	2		17
Mesodon perigraptus Pilsbry	1		1	2	4
Philomycus carolinensis flexuolaris Rafinesque		21		23	44
Retinella indentata (Say)	31	5	12	3	51
Retinella sculptilis (Bland)	14	2	6	2	24
Stenotrema fraternum (Say)	31	27	3	2	63
Stenotrema hirsutum (Say)	19	47	30	27	123
Strobilops aenea Pilsbry	1				1
Triodopsis albolabris (Say)	25	10	19	9	63
Triodopsis hopetonensis (Shuttleworth)			1		1
Triodopsis notata (Deshayes)				3	3
Triodopsis tridentata (Say)	92	34	26	13	165
Triodopsis tridentata edentilabris (Pilsbry)	5	1	1		7
Ventridens intertextus (Binney)	150	27	138	30	345
Ventridens intertextus eutropis Pilsbry			1		1
Zonitoides arboreus (Say)	8		7		15
Totals	595	219	404	156	1374
	814		560		

The weather apparently influences the abundance of snails. The summer of 1952 was very hot and dry compared with the preceding summer. The pH was then generally lower and the temperature higher where collections were made. Apparently the abundance of snails varies somewhat from year to year with conditions of temperature, moisture, and available food. Graduate students who worked in the forest in past years reported species as common which the writer rarely encountered.

Atkins and Labour (1923) found snails in England to be more numerous where pH was 7 to 8. Those with thin shells were more often found where soil tended toward acidity.

SUMMARY

Twenty-one species of land snails were collected in Duke Forest. Thirteen were more common at high levels, seven at low levels, and one was approximately equal in numbers at both levels. Factors that limit the abundance of land snails are character of soil, nature of soil cover, availability of moisture and food, and temperature. Limestone outcrops appear to favor snails. Pine stands support few or no snails; broad-leaf stands are more favorable habitats.

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HEREDITARY ABNORMALITIES IN TOBACCO: ADDENDUM

A paper entitled "Hereditary Abnormalities in Tobacco," by F. A. Wolf and D. G. Sharp, was published in this Journal in June, 1952 (Jour. Elisha Mitchell Sci. Soc. **68**: 85–92). The following references should have been included in the list of literature cited:

SCHIMPER, C. 1851. Besondere Blattbildungen. Flora. 73–78.

STOREY, H. H. 1931. A new virus disease of the tobacco plant. Nature **128**: 187–188.

THUNG, T. H. 1932. De Krul en Kroepoek-ziekten van Tabak en de Oorzakten van hare Verbreiding. Proefstat. Vorstenland. Tabak Meded. **72**: 54 pp.

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TROTTER, A. 1939. Una interessante mutazione teratologica nel Tabacco "Aya Soluk' affine al kroepoek ricontrata nel Leccese. Boll. Tecn. Scofati. 193–202.

WOLF, F. A. 1935. Tobacco Diseases and Decays. xix + 454 pp. Duke University Press, Durham, N. C.

WORSDELL, W. C. 1915, 1916. The Principles of Plant-Teratology. **1**: 270 pp; **2**: 291 pp. London.

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(5) BRAUER, A., AND N. MACON. Amer. Jour. Math. **71:** 349–361. 1949.

BAILEY, JOSEPH R., AND DAVID G. FREY. 1951. Darters of the genus *Hololepis* from some natural lakes of North Carolina. Jour. Elisha Mitchell Sci. Soc. **67:** 191–203.

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